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ORIGINAL ARTICLE

An *in vitro* study of the effect of some dietary components on calculus formation: regulation of calcium phosphate precipitation

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OBJECTIVE: We studied the effects of food components on the *in vitro* formation of calcium phosphate precipitates.

MATERIALS AND METHODS: The effects of food components, such as starch, soybean flour, fish meal, rapeseed oil, and coconut oil, on calcium phosphate precipitation were studied using a pH drop method.

RESULTS: Although the addition of starch had no effect on the rate of precipitation of amorphous calcium phosphate (ACP), it increased both the rate of transformation of ACP to hydroxyapatite (HAP) and the induction time (i.e. time for the initiation of transformation of ACP to HAP to occur); this was irrespective of the heat treatment of the starch. Amylopectin (insoluble constituent of starch) was effective in increasing the rate of HAP transformation, but amylose (soluble constituent of starch) was not. Oil specimen obtained from rapeseed (400 μl ml^{-1}) increased the entire reaction of calcium phosphate precipitation, but that from coconut did not. Protein food, such as soybean flour and fish meal, decreased the rate of transformation of ACP to HAP and increased the induction time, while they had no effect on the rate of ACP precipitation.

CONCLUSION: These results suggest that carbohydrate and oil (both are staple diets for the humans) enhance oral calcification (dental calculus formation or re-mineralization of tooth enamel), while side dishes of protein food would decrease it.

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Keywords: calcium phosphate precipitation; oral calcification; starch; protein food; plant oil

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Introduction

Among the effects of daily food on oral diseases, those of dietary carbohydrates have been extensively investigated in relation to dental caries (Sreebny, 1982; Lingström *et al*, 2000). Sugars can be readily fermented by a wide variety of plaque bacteria to organic acids which could cause caries formation. Among sugars, sucrose is unique in that it serves as a specific substrate for bacterial synthesis of extracellular polysaccharides (glucans). The dental calculus is a calcified dental plaque composed primarily of calcium phosphate mineral salts. Its formation and prevalence have been claimed to be associated with the periodontal disease (Mandel and Gaffar, 1986). The level of calculus formation is affected by several factors including age, gender, diet, oral hygiene and diabetes (White, 1997). It has been reported that exogenous factors which may affect calculus formation include dietary components such as silicon (Damen and Ten Cate, 1989; Hidaka *et al*, 1993b). Gaare *et al* (1989) suggested that differences in eating habits and food, particularly the consumption of rice which contains silicon, may account for the difference of oral hygiene between Asian and European populations.

Regarding the mechanism of the study of the experimental formation of calculus, only a few investigators studied the relationship between foods and the promotion of calculus formation. Dating back over 40 years, Baer *et al* (1961) and Baer and White (1967) reported the effects of starch on the experimental calculus formation in rats. They found that several kinds of starch stimulated the calculus formation and that amylopectin (insoluble fraction of starch), rather than amylose (soluble fraction of starch), played a more important role in the formation of calculus. As not much work has been done in this important subject, we undertook this project to investigate the dietary effects on the *in vitro* formation of calcium phosphate precipitation. Our study would help elucidate the dietary influence on the *in vivo* calculus formation.

When phosphate and calcium ions are mixed at pH 7.4, the formation of amorphous calcium phosphate

(ACP) and its transformation to hydroxyapatite (HAP) *in vitro* can be followed by monitoring the pH drop of the mixture (Hidaka *et al*, 1991). The changes occur in two distinct steps. The effectiveness of any agents that affect the calcium phosphate precipitation can be experimentally studied by measuring (i) the rate of pH decrease in the first step, (ii) the onset time of second rapid decrease, and (iii) the rate of pH drop in the second step (Hidaka *et al*, 1991; Okamoto and Hidaka, 1994). Using this pH drop method, we have reported that some compounds and ions are stimulatory (Hidaka and Abe, 1992; Hidaka *et al*, 1993b; Okamoto and Hidaka, 1994), while others are inhibitory or had no effects (Hidaka *et al*, 1991, 1993a, 1994).

In this paper, we studied the effects of food components on the *in vitro* formation of calcium phosphate precipitates. These components were selected from three major nutrients, i.e. starch as carbohydrate, soybean flour and fish meal as protein, and oil of rapeseed and coconut as lipid.

Materials and methods

Chemicals

Corn starch, potato starch, soluble potato starch, rapeseed oil, and coconut oil were purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan). Starch from rice, amylose (from potato starch; a product of Fluka Chemie GmbH, Bucks, Switzerland), amylopectin (from potato starch; a product of Fluka Chemie GmbH), soybean flour, fish meal (powder), and α-amylase (1000–1500 U mg^{-1}) from human saliva (type IX-A) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Other reagents were purchased from ABIOZ Co. Ltd (Osaka, Japan).

Measurements of pH

A pH meter (F-21; Horiba, Tokyo, Japan) with a combination-type pH electrode (6378-10D; Horiba) and a recorder were used for pH measurements. The final volume of assay solution was 2 ml (Hidaka *et al*, 1991; Okamoto and Hidaka, 1994). The reaction mixture was stirred continuously during the pH measurement, and the temperature was maintained at 37 ± 0.1°C.

Assay of ACP formation, its transformation to HAP, and the induction time by the pH drop method

Both stock solutions, 100 mM $Ca(NO_3)_2$ and 100 mM KH_2PO_4, were prepared in 2 mM HEPES buffer (pH 7.4). To a 1.88 ml buffer solution (2 mM HEPES; pH 7.4), 60 μl of the calcium stock solution was added. Thereafter, 60 μl of the phosphate stock solution was added to start the reaction. The final concentrations of calcium and phosphate were both 3 mM.

For the study of the effect of a food component, one of the following compounds was added to the reaction buffer (at final concentrations shown in parenthesis) 5 min before the addition of phosphate: Corn starch (0.1–2.0 mg ml^{-1}), rice starch (0.1–2.0 mg ml^{-1}), potato starch (0.1–2.0 mg ml^{-1}), potato starch (soluble; 0.5–2.0 mg ml^{-1}), amylose (0.1–1.0 mg ml^{-1}), amylopectin (0.1–2.0 mg ml^{-1}), soybean flour (0.1–0.5 mg ml^{-1}), fish meal (0.5–2.0 mg ml^{-1}), rapeseed oil (100–400 μl ml^{-1}), and coconut oil (100–200 μl ml^{-1}). For the test of heat-treated compounds, such as 2.0 mg ml^{-1} of corn starch, 2.0 mg ml^{-1} of rice starch, 2.0 mg ml^{-1} of potato starch, 2.0 mg ml^{-1} of potato starch (soluble), 0.5 mg ml^{-1} of soybean flour, 2.0 mg ml^{-1} of fish meal, 200–400 μl ml^{-1} of rapeseed oil, or 200 μl ml^{-1} of coconut oil was added. We also tested hydrolysates of potato starch (2.0 mg ml^{-1}), maltose (5.0–10.0 mg ml^{-1}) or α-amylase protein (0.005 mg ml^{-1}). In the experiments involving plant oils, the volume for the measurement increased considerably by the addition of the specimens. Therefore, in the experiments with plant oil, the final concentrations of both calcium and phosphate were increased accordingly in order to compensate that increase.

Three measurements were carried out: (i) the rate of precipitation of ACP as p.p.m. Ca^{2+} min^{-1}, (ii) the rate of transformation of ACP to HAP as p.p.m. Ca^{2+} min^{-1}, and the induction time which is the time between the start of the reaction and the commencement of transformation to HAP in minutes. Both the precipitation of ACP and its transformation to HAP were measured by recording the pH decrease. The rate of pH decrease was converted to the rate of consumption of calcium (parts 10^{-6} min^{-1}) (Hidaka *et al*, 1991). The induction time was determined according to the method of Blumenthal *et al* (1975).

Furthermore, the coefficients of variation calculated from this method were below ~10%.

Heat treatment of starch, soybean flour, fish meal, and oil

Corn starch (2.0 mg ml^{-1}), rice starch (2.0 mg ml^{-1}), potato starch (2.0 mg ml^{-1}), potato starch (soluble; 2.0 mg ml^{-1}), soybean flour (0.5 mg ml^{-1}), or fish meal (0.5 mg ml^{-1}) was suspended in 2 mM HEPES buffer (pH 7.4) and was heated at 97°C for 5 min. Rapeseed oil and coconut oil were heated at 180°C for 5 min. They were used after cooling.

Hydrolysis of starch

A potato starch solution (2.0 mg ml^{-1}) in 2 mM HEPES buffer (pH 6.9) containing 6 mM NaCl was mixed with 0.005 mg ml^{-1} α-amylase (5–7.5 U) and incubated at 37°C for 30 min. One unit of α-amylase liberates 1.0 mg maltose from the starch solution in 3 min at pH 6.9 at 20°C.

Statistics

Data were obtained from three to five measurements and expressed as the mean ± standard deviation. Statistical comparisons were made by ANOVA and Scheffe's test using a statistics software program. $P < 0.05$ was considered significant.

Results

Effects of starch from corn, rice, and potato

As shown in Table 1, at the concentration range of 0.1–0.5 mg ml^{-1}, corn starch had no effects on the *in vitro*

Table 1 Effects of starch from different sources on amorphous calcium phosphate (ACP) formation (p.p.m. Ca^{2+} min^{-1}), its transformation to hydroxyapatite (HAP) (p.p.m. Ca^{2+} min^{-1}), and induction time (min)

Starch	*Concentration used (mg ml^{-1})*	*Ca^{2+} precipitation (p.p.m. min^{-1})*		*Induction time (min)*
		ACP	*HAP*	
None	0	123 ± 12	13.0 ± 1.2	14.8 ± 1.4
Corn	0.1	123 ± 12	13.4 ± 1.2	14.5 ± 1.4
	0.5	123 ± 13	15.5 ± 1.3	17.8 ± 1.6[a]
	2.0	124 ± 12	16.6 ± 1.4[a]	18.4 ± 1.7[a]
Heated	2.0	124 ± 12	29.4 ± 2.5[a,b]	26.6 ± 2.5[a,b]
Rice	0.1	123 ± 12	13.0 ± 1.2	15.9 ± 1.5
	0.5	123 ± 12	12.5 ± 1.1	19.6 ± 1.7[a]
	2.0	125 ± 12	15.6 ± 1.3[a]	21.0 ± 1.9[a]
Heated	2.0	124 ± 12	25.7 ± 2.4[a,b]	29.3 ± 2.5[a,b]
Potato	0.1	124 ± 12	13.0 ± 1.2	15.4 ± 1.4
	0.5	121 ± 13	14.3 ± 1.2	16.1 ± 1.4
	2.0	123 ± 12	15.9 ± 1.3[a]	17.8 ± 1.5[a]
Heated	2.0	124 ± 12	16.0 ± 1.4[a]	36.6 ± 3.3[a,b]
Potato (soluble)	0.5	123 ± 12	13.0 ± 1.2	16.4 ± 1.5
	2.0	125 ± 12	15.5 ± 1.1[a]	20.4 ± 1.7[a]
Heated	2.0	122 ± 11	34.7 ± 3.0[a,b]	42.8 ± 4.0[a,b]

Each value represents mean ± s.d. ACP formation, its transformation to HAP, and induction time were measured by the pH drop method. The concentration of calcium and phosphate were 3 mM each. The test specimens were added to the reaction mixture 5 min before the addition of 3 mM phosphate. Each suspension of additives was heated at 97°C for 5 min in 2 mM HEPES buffer (pH 7.4). The final volume of assay solution, which contains 2 mM HEPES buffer (pH 7.4), was 2 ml. The reaction mixture was stirred at 37 ± 0.1°C. The values for Ca^{2+} precipitation were obtained by converting the rate of pH drop by the method described previously (Hidaka *et al*, 1991).

[a]Significant difference ($P < 0.05$) when compared with the control (no addition).

[b]Significant difference ($P < 0.05$) between experiments with and without heat-treated additives.

formation of calcium phosphate precipitates, except that it increased the induction time 1.2 times at the concentration of 0.5 mg ml^{-1}. At the concentration of 2.0 mg ml^{-1}, it increased both the rate of transformation of ACP to HAP by 28% and the induction time 1.2 times, while it had no effect on the rate of ACP formation. Heat-treated corn starch (2.0 mg ml^{-1}) had greater effects than the non-treated one. It increased both the rate of transformation of ACP to HAP by 126% and the induction time 1.8 times. At the concentration range of 0.1–0.5 mg ml^{-1}, rice starch had no effect both on the rate of ACP formation and on the rate of transformation of ACP to HAP, except that it increased the induction time 1.3 times at the concentration of 0.5 mg ml^{-1}. At the concentration of 2.0 mg ml^{-1}, rice starch had no effect on the rate of ACP formation, but increased both the rate of transformation of ACP to HAP by 20% and the induction time 1.4 times. Heat-treated rice starch (2.0 mg ml^{-1}) had greater effects than the non-treated one. It increased both the rate of transformation of ACP to HAP by 98% and the induction time 2.0 times, while it had no effects on the rate of ACP formation. At the concentration range of 0.1–0.5 mg ml^{-1}, potato starch had no effect on the rate of ACP formation, the rate of transformation of ACP to HAP, and the induction time. At the concentration of 2.0 mg ml^{-1}, it increased both the rate of transformation of ACP to HAP by 22% and the induction time 1.2 times, while it had no effect on the rate of ACP formation. Heat-treated potato starch (2.0 mg ml^{-1}) had greater effect on the induction time than the non-treated one. It increased both the rate of transformation of ACP to HAP by 23% and the induction time 2.5 times. At the concentration range of 0.5 mg ml^{-1}, potato starch (soluble) had no effect on the rate of ACP formation, the rate of transformation of ACP to HAP, and the induction time. At the concentration of 2.0 mg ml^{-1}, it increased both the rate of transformation of ACP to HAP by 19% and the induction time 1.4 times, while it had no effect on the rate of ACP formation. Heat-treated potato starch (soluble; 2.0 mg ml^{-1}) had greater effect on the induction time than the non-treated one. It increased both the rate of transformation of ACP to HAP by 167% and the induction time 2.9 times.

Effects of hydrolysis by α-amylase

As shown in Table 2, the stimulatory effect of potato starch on the rate of transformation of ACP to HAP was abolished by hydrolyzing it with α-amylase (0.005 mg ml^{-1}) prior to the addition. Maltose (5.0–10.0 mg ml^{-1}) or α-amylase alone did not have any affect.

Effects of amylose and amylopectin from potato starch

As shown in Table 3, at the concentration of 0.1 mg ml^{-1}, amylose, one of the starch constituents, increased the induction time 1.3 times. At the concentration of 0.5 mg ml^{-1}, it decreased the rate of transformation of ACP to HAP by 55% and increased the induction time 2.7 times. At the concentration of 1.0 mg ml^{-1}, it drastically reduced the rate of transformation of ACP to HAP and increased the induction time, without affecting the rate of ACP formation. On

Table 2 Effects of potato starch after hydrolysis by α-amylase on amorphous calcium phosphate (ACP) formation (p.p.m. Ca^{2+} min^{-1}), its transformation to hydroxyapatite (HAP) (p.p.m. Ca^{2+} min^{-1}), and induction time (min)

Additives	*Concentration used (mg ml^{-1})*	*Ca^{2+} precipitation (p.p.m. min^{-1})*		*Induction time (min)*
		ACP	*HAP*	
None	0	123 ± 12	13.0 ± 1.3	14.8 ± 1.4
Starch	2.0	124 ± 12	16.0 ± 1.4[a]	36.6 ± 3.3[a]
Starch treated with α-amylase	2.0	127 ± 12	13.2 ± 1.4[b]	14.7 ± 1.4[b]
Maltose	5.0	124 ± 12	12.8 ± 1.1	13.7 ± 1.3
	10.0	124 ± 12	14.0 ± 1.4	13.7 ± 1.3
α-Amylase alone	0.005	127 ± 12	12.6 ± 1.2	13.1 ± 1.3

Each value represents mean ± s.d. Details are the same as those shown in Table 1.
[a]Significant difference ($P < 0.05$) when compared with the control (no addition).
[b]Significant difference ($P < 0.05$) between treatment and non-treatment with α-amylase.

Table 3 Effects of amylose and amylopectin from potato starch on amorphous calcium phosphate (ACP) formation (p.p.m. Ca^{2+} min^{-1}), its transformation to hydroxyapatite (HAP) (p.p.m. Ca^{2+} min^{-1}), and induction time (min)

Additives	*Concentration used (mg ml^{-1})*	*Ca^{2+} precipitation (p.p.m. min^{-1})*		*Induction time (min)*
		ACP	*HAP*	
None	0	123 ± 12	13.0 ± 1.3	14.8 ± 1.4
Amylose	0.1	124 ± 12	14.8 ± 1.4	19.6 ± 1.7[a]
	0.5	123 ± 13	5.88 ± 0.5[a]	40.0 ± 3.7[a]
	1.0	124 ± 12	ND	< 100
Amylopectin	0.1	123 ± 12	11.3 ± 1.1	25.3 ± 2.3[a]
	0.5	123 ± 12	23.9 ± 2.3[a]	36.6 ± 3.3[a]
	2.0	124 ± 12	24.6 ± 2.4[a]	41.7 ± 3.9[a]

Each value represents mean ± s.d. Details are the same as those shown in Table 1. Amylose (10 mg ml^{-1}) was solubilized with 1.0 ml of 0.5 N NaOH. Then, the pH of the solution was adjusted to pH 7.4 with HCl. Amylopectin was usually solubilized by 2 mM HEPES buffer (pH 7.4). ND, not determined.
[a]Significant difference ($P < 0.05$) when compared with the control (no addition).

Table 4 Effects of soybean flour and fish meal on amorphous calcium phosphate (ACP) formation (p.p.m. Ca^{2+} min^{-1}), its transformation to hydroxyapatite (HAP) (p.p.m. Ca^{2+} min^{-1}), and induction time (min)

Proteins	*Concentration used (mg ml^{-1})*	*Ca^{2+} precipitation (p.p.m. min^{-1})*		*Induction time (min)*
		ACP	*HAP*	
None	0	123 ± 12	13.0 ± 1.3	14.8 ± 1.4
Soybean	0.1	123 ± 13	6.91 ± 0.6[a]	23.2 ± 2.1[a]
Flour	0.25	124 ± 12	5.69 ± 0.5[a]	35.8 ± 3.4[a]
	0.5	121 ± 13	3.41 ± 0.3[a]	57.4 ± 5.0[a]
Heated	0.5	123 ± 12	2.76 ± 0.2[a, b]	75.5 ± 6.5[a, b]
Fish meal	0.5	123 ± 13	11.9 ± 1.0	16.8 ± 1.5
	1.0	124 ± 12	9.50 ± 0.8[a]	21.0 ± 2.0[a]
	2.0	114 ± 11	6.76 ± 0.6[a]	28.1 ± 2.7[a]
Heated	2.0	112 ± 10	5.89 ± 0.5[a]	29.1 ± 2.8[a]

Each value represents mean ± s.d. Details are the same as those shown in Table 1.
[a]Significant difference ($P < 0.05$) when compared with the control (no addition).
[b]Significant difference ($P < 0.05$) between with and without heat treatment (see text for details).

the contrary, amylopectin, another constituent of starch, increased the induction time 1.7 times at the concentration of 0.1 mg ml^{-1}. However, at the concentration range of 0.5–2.0 mg ml^{-1}, it increased both the rate of transformation of ACP to HAP by 80–90% and the induction times 2.5–2.8 times.

Effects of soybean flour and fish meal

As shown in Table 4, at the concentrations of 0.1, 0.25 and 0.5 mg ml^{-1}, soybean flour decreased the rate of transformation of ACP to HAP, by 47%, 56%, 74%, respectively, and increased the induction time, 1.6, 2.4, 3.9 times, respectively. However, it had no effect on the rate of ACP formation. Heat treatment of soybean flour (0.5 mg ml^{-1}) enhanced the effects; it decreased the rate of HAP transformation by 79% and increased the induction time 5.1 times. At the concentration of 0.5 mg ml^{-1}, fish meal had no effects on calcium phosphate precipitation. However, at the concentration range of 1.0–2.0 mg ml^{-1}, it decreased the rate of

Table 5 Effects of plant oil on amorphous calcium phosphate (ACP) formation (p.p.m. Ca^{2+} min^{-1}), its transformation to hydroxyapatite (HAP) (p.p.m. Ca^{2+} min^{-1}), and induction time (min)

Oil	*Concentration used (μl ml^{-1})*	*Ca^{2+} precipitation (p.p.m. min^{-1})*		*Induction time (min)*
		ACP	*HAP*	
None-1	0	133 ± 12	14.2 ± 1.4	14.4 ± 1.4
Rapeseed	100	149 ± 13	16.0 ± 1.5	14.2 ± 1.4
None-2	0	146 ± 13	15.9 ± 1.5	14.0 ± 1.3
Rapeseed	200	160 ± 15	17.7 ± 1.3	13.4 ± 1.3
Heated	200	132 ± 12[b]	13.5 ± 1.5[b]	13.9 ± 1.3
None-3	0	209 ± 20	21.0 ± 2.0	11.0 ± 1.0
Rapeseed	400	286 ± 27[a]	32.3 ± 3.1[a]	6.14 ± 0.5[a]
Heated	400	192 ± 17[b]	19.9 ± 1.7[b]	12.0 ± 1.1[b]
None-1	0	133 ± 12	14.2 ± 1.4	14.4 ± 1.4
Coconut	100	129 ± 13	13.5 ± 1.3	15.2 ± 1.4
None-2	0	146 ± 13	15.9 ± 1.5	14.0 ± 1.3
Coconut	200	157 ± 15	17.7 ± 1.6	15.7 ± 1.5
Heated	200	163 ± 13	18.2 ± 1.5	14.0 ± 1.4

Each value represents mean ± s.d. Details are the same as those shown in Table 1.
[a]Significant difference ($P < 0.05$) when compared with respective controls (no addition; None-1, None-2, None-3).
[b]Significant difference ($P < 0.05$) between with and without heat treatment (see text for details).

transformation of ACP to HAP by 26–61% and increased the induction time 1.4–1.8 times, while it had no effect on the rate of ACP formation. At the concentration of 2.0 mg ml^{-1}, the effect of heat-treated fish meal on both the rate of transformation of ACP to HAP and on the induction time was similar to that of the non-heat treated one.

Effects of oil from rapeseed and coconut

As shown in Table 5, at the concentration range of 100–200 μl ml^{-1}, rapeseed oil emulsion had no effects on *in vitro* formation of calcium phosphate precipitates. At the concentration of 400 μl ml^{-1}, it increased both the rate of ACP formation by 37% and the rate of transformation of ACP to HAP by 54%, while it shortened the induction time by 44%. Heat-treated rapeseed oil (200 and 400 μl ml^{-1}) had no effect on the rate of ACP formation, the rate of transformation of ACP to HAP and the induction time. They had lesser effects than the non-treated oil. At the concentration range of 100–200 μl ml^{-1}, coconut oil emulsion had no effect on *in vitro* calcium phosphate precipitation. Heat-treated coconut oil (200 μl ml^{-1}) had no effect, either.

Discussion

Starch is a major component of the human diet. In Asia, starch can be found in a wide variety of foods, particularly rice, and constitutes a high percentage of total dietary carbohydrates. In Japan, rice starch occupies 69% of total carbohydrates in 2002 (Ministry of Health, Labour and Welfare of Japan, 2002). As starch increased the rate of transformation of ACP to HAP in the calcium phosphate precipitation (Table 1), it is possible that starch enhances calculus formation. Heat treatment of raw starch in daily cooking changes the starch structure from micellary β- to α-type, which is easily digestible for the human being (Lingström *et al*, 2000). As the heat treatment of corn and rice starch increased the rate of transformation of ACP to HAP 1.8–1.6 times, it seems that the formation of calculus could be more enhanced by cooking. However, Mörmann and Mühemann (1981) reported that in the human mouth, α-type starch can be hydrolyzed by both salivary and bacterial amylase into maltose, maltotriose, and low-molecular weight dextrin, all of which are good sources for bacterial acid production (Kashket *et al*, 1994). As the stimulatory effects of starch were abolished by the actions of α-amylase, and as maltose did not affect the formation of calcium phosphate precipitates at higher concentrations (Table 2), it is possible that the action of salivary and bacterial amylase could weaken and abolish the stimulatory effect on calculus formation. However, the concomitant acid production would promote the occurrence of caries. Thus, there is an inverse correlation between calculus formation and occurrence of dental caries (see the scheme shown in Figure 1).

Amylopectin (insoluble fraction) increased the rate of transformation of ACP to HAP similar to the action of starch, while amylose (soluble fraction) decreased it (Table 3). This indicates that the stimulatory effect by

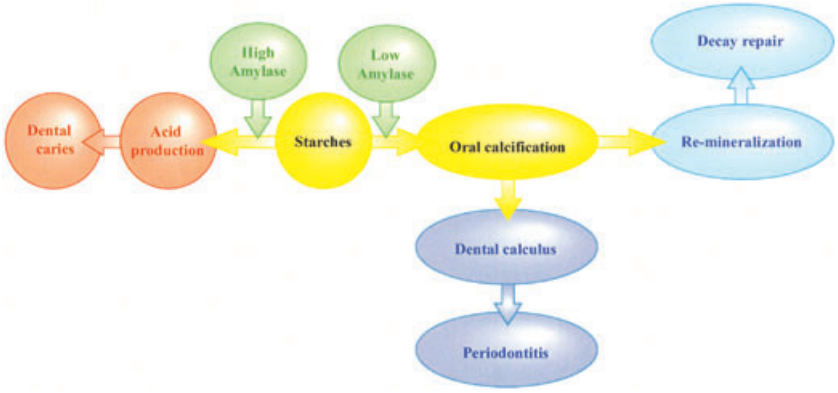

Figure 1 Proposed mechanisms on the action of amylase in oral diseases. When hydrolysis by the salivary and bacterial amylase does not occur, the reaction proceeds toward the right-hand side, resulting in oral calcification (calculus formation or re-mineralization). In this case, calculus formation causes periodontitis and the re-mineralization repairs of the decay in dental caries. On the contrary, when the hydrolysis by amylase occurs extensively, the reaction proceeds toward the left-hand side, resulting in acid production followed by dental caries

starch may be caused by its amylopectin fraction. The stimulation by amylopectin may be associated with its insoluble, sticky property (Baer and White, 1967). The inhibitory effects of amylose may be ascribed to its soluble and non-sticky properties. The stimulatory ability of potato starch was weakest among all types of starch. In an experiment using rats, Baer and White (1967) revealed that the stimulatory effects on calculus formation by unmodified potato starch was the weakest and the order of stimulatory effect was: unmodified corn starch > unmodified rice starch > unmodified potato starch. They also showed that corn amylopectin formed more calculus than corn amylose. Their *in vivo* results of both stimulatory effects by starch of corn, rice and potato and those by amylopectin (but not by amylose) were reproduced in our *in vitro* experiments. Heat-treated potato starch did not increase the rate of transformation of ACP to HAP, but heat-treated soluble potato starch did, to a great extent (Table 1). Therefore, it seems that no effect with heat-treated potato starch may be related to the lesser solubility of this type of potato starch.

Hunter *et al* (1986) reported that collagen, gelatin, and agarose gels promoted HAP precipitation depending on the rate of diffusion, but that the reaction did not reach a steady state. TenHuisen and Brown (1992) reported that gelatin influenced the kinetics of gypsum formation and its microstructure. Macromolecular adsorption was shown to have significant effects on both crystal growth and dissolution of HAP (TenHuisen and Brown, 1994). Therefore, the stimulatory effect of starch on the rate of transformation of ACP to HAP may be caused by its adsorption onto calcium phosphate crystals. A similar mechanism has been reported for gelatin (Hidaka and Liu, 2003). Stimulatory effects of oxidized lauryl sulfate (Hidaka and Abe, 1992), silica, and clay minerals (Hidaka *et al*, 1993b), and a few cathionic metal ions (Okamoto and Hidaka, 1994) shortened the induction time. Similar to both starch and gelatin, fluoride increased the rate of transformation of ACP to HAP, but it increased induction time (Hidaka and Okamoto, 2003). The stimulatory effect caused by the adsorption of an anionic fluoride at active growth sites (Meyer and Nancollas, 1972) was also observed in our experiment with starch and gelatin.

Another dietary stimulator for calcium phosphate precipitation was oil specimen obtained from rapeseed. At the concentration of 400 μl ml^{-1}, rapeseed oil increased the rates of both ACP formation and its transformation to HAP (Table 5). It is known that rapeseed oil contains greater amount of unsaturated fatty acids [88% (w/w)], while coconut oil contains greater amount of saturated fatty acids [85% (w/w)] (Kagawa, 2005). Heat treatment of rapeseed oil had lesser effects than non-treated ones in the range of 200–400 μl ml^{-1}. On the other hand, both heat-treated coconut oil and non-heat-treated oil had no effect. It is known that heat treatment causes more oxidative damages in unsaturated fatty acids than in saturated fatty acids. Therefore, both the stimulatory effect of non-heat-treated rapeseed and its inhibitory effect after heat treatment may be related with its higher content of unsaturated fatty acids.

The pH value of a 400 μl ml^{-1} emulsion of coconut oil could not be measured because the measurement became unstable. The stimulatory effects of rapeseed oil (400 μl ml^{-1}) were observed over the entire period of calcium phosphate precipitation (Table 5). It is possible that the physical property of an oil emulsion may crucially influence the process of formation of calcium phosphate precipitates. This reaction might proceed in a transient emulsion state which is formed by continuous stirring. If this is the case, the hydrophilic surface of oil droplets in the emulsion would serve as the nuclei (templates) for the formation of new crystals (similar to the membranous layer of the matrix vesicle; Anderson, 2003).

Contrary to the stimulatory effects of starch and oil, protein components, such as soybean flour and fish meal, had a remarkable inhibitory activity on the formation of calcium phosphate precipitates (Table 4). The inhibitory effects of proteins and body fluid, e.g., bovine serum albumin, casein, phosvitin, and saliva on calcium phosphate precipitation have been demonstrated (Hidaka *et al*, 1991, 2004; Hidaka, 2003). It seems that the adsorption of proteins onto calcium phosphate crystals suppresses crystal growth.

There is a tendency to regard calculus formation as a negative factor in oral hygiene. However, the event of oral calcification includes calculus formation as well as the re-mineralization of tooth enamel, which is a protective reaction against the decay by dental caries. Although there is no clear correlation between calculus formation and dental caries, Frostell and Baer (1971) summarized that very high calculus scores and very low caries scores were found in animals fed unmodified starch. They added that a tendency of high scores of both calculus and caries was found in animals fed pregelatinized starch. It is possible that pregelatinized starch increased the occurrence of caries, because this is readily fermented by plaque bacteria when compared with unmodified starch. The inverse correlation between calculus formation and occurrence of caries is related to a common clinical belief that humans with high caries activity have little tendency to form calculus, and *vice versa* (White and Russell, 1962; Tanzer *et al*, 1993).

As schematically shown in Figure 1, we propose that such an inverse correlation between calculus formation and occurrence of caries is triggered by the action of salivary and bacterial amylases. When the starch hydrolysis by amylase in dental plaque occurs very little or none (low amylase), the reaction is directed toward the right to oral calcification. This is led to either a periodontal disease followed by formation of calculus or re-mineralization which could repair the decay of tooth enamel. On the contrary, when there is extensive hydrolysis of starch, the reaction proceeds toward the left-hand side (Figure 1). Acids were produced followed by the decay of tooth enamel. The hydrolytic activity of α-amylase in the dental plaque may be regulated by the availability of starch (as a substrate), isozymes, chloride ion, and α-amylase-binding oral bacteria (Scannapieco *et al*, 1993). Therefore, it is conceivable that starch would play a regulatory role between oral calcification (enhancing calculus formation or re-mineralizing the

decay of enamel) and occurrence of dental caries (progressing the acid production and the decay of tooth enamel). Starch might help elucidate the inverse correlation between oral calcification and occurrence of caries. In this connection, by modifying the 'silicon theory' proposed by Gaare *et al* (1989), we would like to propose a 'rice starch theory' in stimulating calculus formation. Although our *in vitro* results with food components may not be immediately expanded to oral calcification *in vivo*, our data would shed light on the reaction pathways of dental calcification and its regulation mechanism.

In conclusion, we studied the effects of food components from major nutrients, i.e., starch, soybean flour, fish meal, rapeseed oil, and coconut oil, and on calcium phosphate precipitation using a pH drop method. Starch and rapeseed oil stimulated, whereas protein nutrients inhibited, calcium phosphate precipitation (calculus formation or re-mineralization of tooth enamel).

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