Effective fractions of a Chinese traditional (Kampo) medicine, Rikko-san and its herbal constituents on the formation of calcium phosphate precipitates

Saburo HIDAKA,*[a] Yoshizo OKAMOTO,[b] Kimio ABE,[a] Mamoru SUEKAWA[c] and Sheng Yan LIU[c]

[a]*Department of Oral Biochemistry,* [b]*Department of Dental Materials, Fukuoka Dental College,* [c]*Tsumura & Co.*

(Received March 1, 1994. Accepted July 7, 1994.)

Abstract

Since traditional Chinese (Kampo) medicines are potential preventors of dental calculus, the inhibitory effects of Rikko-san (Kampo medicine) and its five herbal constituents (Saishin, Shoma, Bofu, Ryutan, and Kanzo) on the formation of calcium phosphate precipitates were studied. Rikko-san had an activity about 1/67 of that of 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP) in terms of the activity per weight. The inhibitory effects of Shoma, Saishin, and Kanzo on both decreasing the rate of hydroxyapatite (HAP) transformation and increase in the induction time are comparable to those of Rikko-san.

The membrane filtrates (limiting 3 kDa) of Rikko-san and its five herbal constituents showed the inhibitory effects on the rate of HAP transformation similar to their original solutions. However, these activities to extend the induction time were much reduced. In these filtrates, the recoveries of polyphenols were 50-70%. In contrast, the filtrates of Rikko-san and its five herbal constituents through a membrane limiting 10 kDa had similar activities and similar polyphenol contents as those of corresponding original solutions. Therefore, the fraction below 3 kDa contains most of the substances which are effective for the inhibition of the rate of HAP transformation, whereas compounds with their molecular weights between 3 and 10 kDa may be responsible for extending the induction time.

Key words Kampo medicines, herbs, polyphenols, anti-calculus agents.

Introduction

It has been suggested that dental calculus can contribute to the progression of gingivitis or periodontal diseases because its porous structure can retain potentially toxic substances including stimulators of bone resorption.[1] In recent years, there has been considerable interest in synthetic agents, *i.e.*, sodium etidronate,[2] zinc salts,[3] an oligomer of sulfacrylic acid[4] and editempa,[5] which are useful in the prevention of calculus.[6] However, natural products such as an anticalculus agent have not been developed.

Recently a few attempts were made to extend the efficacy of Chinese traditional (Kampo) medicines beyond their traditional indications and to apply them to oral diseases. For example, Namba *et al.*[7] have reported the anticariogenic potentials of Kampo medicines. We also performed an *in vitro* screening of twenty-three kinds of Kampo medicines on their inhibitory effects for the formation of calcium phosphate precipitates and found that nine medicines including Rikko-san may have potential as anticalculus agents.[8]

As an extension of our previous investigation, we studied *in vitro* inhibitory effects of Rikko-san and its five herbal constituents on calcium phosphate precipitation. Furthermore, in order to seek effective substances, we carried out a fractionation study of Rikko-san and its herbal constituents. In this report, we

〒814-01 福岡市早良区田村2丁目15番1号
福岡歯科大学口腔生化学講座　日高三郎
15-1, Tamura 2-Chome, Sawara-Ku, Fukuoka 814-01, Japan

和漢医薬学雑誌 **11**, 106－113, 1994

present data on the inhibitory effects of Rikko-san and its five herbal constituents, *i.e.*, Saishin, Shoma, Bofu, Ryutan, and Kanzo on the formation of calcium phosphate and its transformation. Rikko-san is one of a few Kampo medicines which are used in curing dental diseases, *i.e.*, tooth pain and stomatitis.[9]

Materials and Methods

Kampo medicines and chemicals : Dried powders of Rikko-san and herbs were supplied by Tsumura & Co., (Tokyo, Japan). Rikko-san contains five herbal constituents, *i.e.*, 23.5% (w/w) Saishin (JP (Japanese Pharmacopeia), *Asiasari Radix*), 23.5% (w/w) Shoma (JP, *Cimicifugae Rhizoma*), 23.5% (w/w) Bofu (JP, *Saposhnikoviae Radix*), 12% (w/w) Ryutan (JP, *Gentianae Scabrae Radix*) and 18% (w/w) Kanzo (JP, *Glycyrrhizae Radix*) and is claimed to be effective in killing tooth pain.[8, 9] Ethyleneglycol-bis-(β-aminoethylether) N,N'-tetra-acetic acid (EGTA) was purchased from Sigma Chemicals (St Loius, MO, U.S.A.). A 60% solution of a 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) was purchased from Tokyo Kasei (Tokyo, Japan). Tannic acid (from Chinese nutgalls) were purchased from Katayama Chemicals (Osaka, Japan). All other reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Assays of amorphous calcium phosphate (ACP) formation and transformation to hydroxyapatite (HAP) by the pH drop method : The formation of ACP and the transformation to HAP were measured by the pH drop method.[10] In brief, a pH meter (F-7, Horiba, Japan) with a pH electrode (6028-10T, Horiba, Japan) and a recorder were used for pH measurements.

Two stock solutions, 100 mM $Ca(NO_3)_2$ and 100mM KH_2PO_4 were prepared in 2 mM Hepes buffer (pH 7.4). To a 1.88 ml of 2 mM Hepes buffer, 60 μl of the calcium stock solution was added. Then, 60 μl of the phosphate stock solution was added to start the reaction. The final concentrations of calcium and phosphate were both 3 mM. The final volume of the reaction mixture was 2 ml. The temperature was maintained at 37±0.1°C and the reaction mixture was stirred continuously.

The formation of ACP and its transformation to HAP *in vitro* occurred in two distinct steps after phosphate (0.75-10 mM) was added to a 3 mM calcium solution at pH 7.4. The reaction was followed acidimetrically by recording the pH changes. The pH decrease followed a characteristic pattern as follows : first, an initial rapid decrease occurring between 1-3 min and, secondly, a rapid decrease occurring between 3 and 16 min. We measured the initial rate of pH drop upon addition of phosphate to a calcium buffer solution. We used the rate as an index of the formation of ACP and its transformation to HAP.

A solution of Rikko-san or one of its five herbal constituents (0.1-0.5mg/ml) was added to the reaction mixture 5 min before the addition of phosphate. HEBP (10-60 μM) or EGTA (0.125-0.5 mM) was added in the same manner. The reaction rates were converted to the rate of consumption of calcium (parts/10^6/min).[10] The induction time was determined according to the method of Blumenthal *et al.*.[11]

Determination of the chelating capacity : The free calcium concentration in Rikko-san or in each of its five herbal solution was determined at 23±0.1°C with a calcium electrode (93-20, Orion Research Incorporated, U.S.A.). A solution containing 3 mM $Ca(NO_3)_2$, 0.08 M KCl and 20 mM Hepes (pH 7.4) was titrated with Rikko-san or one of its five herbal constituents (0.2 and 0.5 mg/ml), and with HEBP (10 and 60 μM) or EGTA (0.5 mM). The changes in free calcium concentration caused by the addition of these solutions were measured.

Estimation of total polyphenol content : The total polyphenol content was measured with the Folin-Ciocalteau reagent.[12] In brief,distilled water (1.0 ml), 0.71 M sodium carbonate (0.8 ml) and sample (0.2 ml) were mixed and 0.05 ml of 2 N Folin-Ciocalteau reagent was added. After incubating for 15 min at 45°C, the absorbance at 765 nm was determined. Tannic acid from Chinese nutgalls was used as the standard.

Fractionation of Kampo medicines and their herbal constituents : The Rikko-san solution (10 mg/ml) was subjected to dialysis using a cellulose tube (MW limits 12-14 kDa) or to filtration through a membrane filter (MW limits 3 or 10 kDa ; Centricon, Amicon Grace Co., U.S.A.). Solutions (10 mg/ml) of five herbs were also filtered through a membrane filter (MW limits 3 or 10 kDa). Effects of these dialysates and filtrates on the *in vitro* formation of

calcium phosphate were assayed as described above.

Statistics : Data were obtained from 3 to 5 measurements, and expressed as the means ± standard deviations. Statistical comparisons were made using ANOVA and Scheffé's Test. The difference was considered significant when $p<0.05$.

Results

Effects of Rikko-san and its herbal constituents on calcium phosphate precipitation

At a concentration of 0.5 mg/ml, Rikko-san and each of its five herbal constituents (Saishin, Shoma, Bofu, Ryutan and Kanzo) had no inhibitory effect on the formation of ACP (Table I). All this medicine and herbs tested showed significant inhibitory effects on both the transformation of HAP (20 - 78 % of the control) and on the increase of the induction time (the ratio to control ranged from 2.0 to 8.2) (Table I).

Calcium chelating activity of Rikko-san and its herbal constituents

The calcium complexion of Rikko-san and its herbal constituents was examined. As shown in Table II, at the concentration of 0.2 mg/ml, Saishin, Shoma, Ryutan and Kanzo showed some chelating activities. At the concentration of 0.5 mg/ml, all of Rikko-san and its five herbal constituents showed chelating capacity, although the differences were not statistically significant.

Effects of HEBP or EGTA on calcium phosphate precipitation and chelation

HEBP had no inhibitory effects on the ACP formation, but showed significant inhibitory effects on both HAP transformation and the induction time at concentrations of 20-60 μM (Table III). HEBP showed some chelating activities in 10 - 60 μM (Table II). EGTA showed a significant inhibitory effect only on HAP transformation at the concentration of 0.25 mM. At 0.5 mM, it showed significant inhibitory effects on ACP formation, HAP transformation and the induction time (Table III).

Effect of the chelating activity on other measurements

As described above, Rikko-san and its five herbal constituents had some calcium chelating ability at the concentration of 0.5 mg/ml. Therefore, we examined

Table I Effects of Kampo medicine and herbal constituents on the formation of amorphous calcium phosphate (ACP) and its transformation to hydroxyapatite (HAP).

Concentration used (mg/ml)		Ca^{2+} consumption (ppm/min) ACP	HAP	Induction time (min)
None	0	125±12.0	15.0 ±1.3	14.8± 1.5
Rikko-san	0.5	133± 5.0	4.79±0.23*	73.9± 4.8*
Saishin	0.5	119± 9.3	5.08±0.20*	60.7± 4.8*
Shoma	0.5	131± 8.5	2.99±0.57*	121 ±11*
Bofu	0.5	125± 6.6	11.7 ±0.82*	30.3± 2.8*
Ryutan	0.5	131± 6.2	9.98±0.49*	46.3± 4.1*
Kanzo	0.5	130± 5.0	4.19±0.20*	48.3± 4.8*

The ACP formation and HAP transformation were measured by the pH drop method. Final concentrations of 3 mM calcium and 3 mM phosphate were used. Rikko-san or its constituents was added to the reaction mixture 5 min before the addition of 3 mM phosphate. The final volume of the assay solution, which contains 2 mM Hepes (pH 7.4) was 2 ml. The reaction mixture was stirred at 37±0.1°C. Values were presented as the rate of consumption of calcium (ppm/min).
*Indicates a significant difference ($p<0.05$).

Table II Chelating capability of Kampo medicine, its herbal constituents and HEBP.

	Concentrations used	Ca^{2+} concentrations (mM)
None	−	3.00±0.015
Rikko-san	0.2 mg/ml	3.00±0.015
	0.5 mg/ml	2.92±0.014
Saishin	0.2 mg/ml	2.97±0.015
	0.5 mg/ml	2.90±0.015
Shoma	0.2 mg/ml	2.97±0.015
	0.5 mg/ml	2.83±0.015
Bofu	0.2 mg/ml	3.00±0.015
	0.5 mg/ml	2.97±0.014
Ryutan	0.2 mg/ml	2.97±0.015
	0.5 mg/ml	2.92±0.014
Kanzo	0.2 mg/ml	2.97±0.015
	0.5 mg/ml	2.83±0.015
HEBP[a]	10 μM	2.97±0.011
	60 μM	2.85±0.011
EGTA[b]	0.5 mM	2.50±0.011*

Calcium solution (3mM) containing 0.08 M KCl and 20 mM Hepes (pH 7.4) was titrated with Rikko-san, its herbal constituents (0.2 and 0.5 mg/ml), or with HEBP (10 and 60 μM) solution. The changes in free calcium ion concentration were measured using a calcium electrode.
[a]Abbreviation of 1-hydroxyethylidene-1,1-bisphosphonate.
[b]Abbreviation of ethyleneglycol-bis-(β-aminoethylether) N,N'-tetraacetic acid.
*Indicates a significant difference ($p<0.05$).

Table III Effects of HEBP and EGTA on the formation of amorphous calcium phosphate (ACP) and its transformation to hydroxyapatite (HAP).

	Concentration used	Ca^{2+} consumption (ppm/min) ACP	HAP	Induction time(min)
None	0	125 ±12	15.0 ±1.3	14.8± 1.5
HEBP[a]	10 μM	123 ± 9.3	9.92±0.61*	18.0± 1.2
	20 μM	123 ± 8.0	7.81±0.60*	34.7± 7.1*
	40 μM	123 ± 8.0	4.61±0.61*	91.8± 8.7*
	60 μM	120 ±10	2.20±0.11*	207 ±13*
EGTA[b]	0.125 mM	119 ± 8.5	13.0 ±1.3	14.6± 1.5
	0.20 mM	122 ±11	11.8 ±1.1	15.1± 1.4
	0.25 mM	110 ± 9.0	9.20±0.61*	16.5± 2.1
	0.5 mM	7.75± 0.54*	6.11±0.41*	23.5± 2.3*

Experimental conditions were the same as those shown in Table I.
[a]Abbreviation of 1-hydroxyethylidene-1,1-bisphosphonate.
[b]Abbreviation of ethyleneglycol-bis-(β-aminoethylether) N,N'-tetraacetic acid.
*Indicates a significant difference ($p<0.05$).

whether this activity would effect calcium phosphate precipitation. The chelating activities of Rikko-san and its five herbal constituents were equivalent to 0.03-0.17 mM EGTA (see Table II). At these concentrations of EGTA, it did not show much inhibitory effects on the calcium phosphate precipitation (Table III); ACP formation, HAP transformation and on the induction time.

Comparison with HEBP

The inhibitory effect on the HAP transformation was compared between HEBP (10-60 μM) and Rikko-san (0.1-0.5 mg/ml). As shown in Table III and Fig. 1A, a 50 % inhibition of HAP transformation was attained with concentrations of 4.5 μg/ml (=22 μM) of HEBP and 0.30 mg/ml of Rikko-san. This indicated that the effectiveness of Rikko-san was about 1/67 of that of HEBP in terms of the activity per weight.

Comparison between Rikko-san and five herbal constituents

To compare the five herbal constituents with Rikko-san, their effects on the HAP transformation (% of control) and the induction time (min) were plotted in Fig. 1A and 1B. The curves for Saishin, Shoma and Kanzo were comparable to Rikko-san. Those for Ryutan and Bofu had a shoulder in their lower concentration range. The range of the inhibitory effects of the five herbal constituents varied widely. At the concentration of 0.5 mg/ml, the order of inhibitory effects of Rikko-san and its five herbs on the HAP transformation (% of control) was Shoma > Kanzo > Rikko-san > Saishin > Ryutan > Bofu. The effects on the induction time (min) was in the order of Shoma > Rikko-san > Saishin > Kanzo=Ryutan > Bofu.

Effective fractions of Rikko-san and herbal constituents which inhibit calcium phosphate precipitation

The dialysate of a Rikko-san obtained through a cellulose membrane (MW limits 12-14 kDa) lost a large portion of its polyphenol content (by 96.4 %; from 485 ±5.2 to 17.5±0.34 μg/ml), and lost its inhibitory activities on both the rate of HAP transformation (from 7.35±0.71 to 15.0±1.4 ppm/min) and the induction time (from 41.4±3.5 to 16.8±1.5 min). As shown in Table IV, the filtrate through a membrane with a 10-KDa cut-off had inhibitory activities on both the rate of HAP transformation (50 % inhibition) and the induction time (2.7 times) similar to the original solution, and had a similar polyphenol content (about 96 % recovery). The filtrate through a membrane with a 3-kDa cut-off showed similar inhibitory effect on the rate of HAP transformation (50 % inhibition) but its effect on the induction time was reduced from 2.8 to 1.9. The polyphenol content was reduced by about 40 %.

The filtrates of five herbal constituents through a membrane with a 10-kDa cut-off had similar inhibitory activities and polyphenol contents (96 - 100 %

recovery) as compared to their original solutions. The filtrates from each of five herbal constituents through a membrane with 3-kDa cut-off showed similar inhibitory effect on the rate of HAP transformation. However, their inhibitory effects on the induction time were much reduced. Namely, when the polyphenol contents of Rikko-san and its five herbs were reduced by 30-50 %, their inhibition on the rate of HAP transformation did not change, but their inhibitory effects on the induction time decreased from 2.8 to 1.8 times in Saishin, 3.9 to 1.8 times in Shoma, 1.5 to 1.2 times in Bofu, 2.2 to 1.4 times in Ryutan and 2.3 to 1.4 times in Kanzo.

Discussion

The 50 % inhibition of the HAP transformation was attained by the concentrations of 4.5 μg/ml of HEBP and 0.30 mg/ml of Rikko-san, respectively (see Fig. 1A). Therefore, by comparing with HEBP, a Kampo medicine, Rikko-san had an inhibitory activity about 1/67 of that of HEBP. HEBP has been proven to be effective in decreasing the dental calculus in the rat and the human,[2, 3] but it may be toxic at the effective dose, because it could affect bone turnover and mineralization.[13] Since Rikko-san is known to have little side effects, it may be used as a safer anticalculus agent.

We have previously shown that Shigyaku-san and Shikunshi-to had inhibitory effects on the rate of HAP transformation which were 1/100 and 1/400 of that of HEBP, respectively.[14] These Kampo medicines which were touted for use in digestive diseases,[8, 9] were shown to be effective in decreasing the deposition of supragingival dental calculus when given orally to the rat.[14] Since the *in vitro* inhibitory effects of Rikko-san was found to be larger than those of Shigyaku-san and Shikunshi-to, Rikko-san may be more efficacious in decreasing *in vivo* calculus formation.

Rikko-san contains Saishin, Shoma, Bofu, Ryutan and Kanzo.[9] Among these five kinds of herbal constituents, Shoma, Saishin and Kanzo constitute 24, 24 and 18 % (w/w) of Rikko-san, respectively.

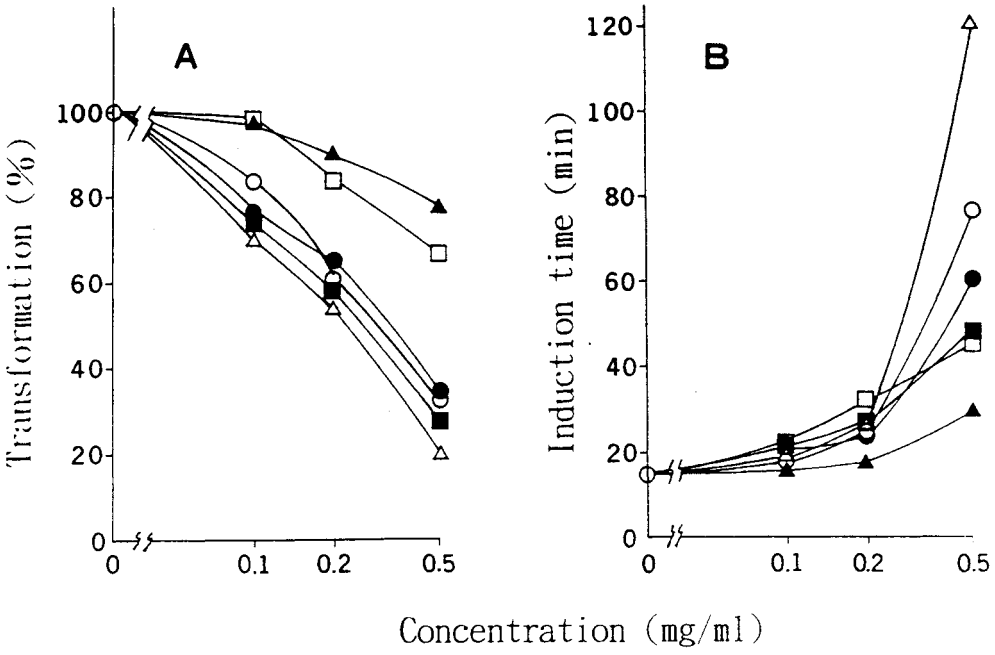

Fig. 1 Inhibitory effects of Kampo medicines on the hydroxyapatite transformation (% of control) (A) and the induction time (B). Kampo medicines: Rikko-san (○), Saishin (●), Shoma (△), Bofu (▲), Ryutan (□) and Kanzo (■).
The experimental conditions were the same as those shown in Table I. Data were obtained from 3 to 5 measurements.

Their inhibitory effects on both decreasing the rate of HAP transformation and increasing the induction time are comparable to those of Rikko-san (Fig. 1A and 1B).

In Rikko-san or in its constituents, some chelating properties were observed when their concentra-

Table IV Polyphenol contents in Kampo medicines and its five herbal constituents, and effects of their filtrates (limiting 3 and 10 kDa) on the ACP formation and HAP transformation.

	Polyphenol contents (μg/ml)	Ca^{2+} consumption (ppm/min)		Induction time (min)
		ACP	HAP	
Control	0	125±12	15.0 ±1.3	14.8±1.5
Rikko-san				
Original solution	489±5.2	127±12	7.35±0.71*	41.4±3.5*
Filtrates (limiting 10 kDa)	469±5.5	125±11	7.57±0.71*	39.3±2.0*
Filtrates (limiting 3 kDa)	287± 3.5**	125±12	7.49±0.83*	27.8±1.5*
Saishin				
Original solution	463± 4.5	123±12	7.53±1.1*	41.3±3.8*
Filtrates (limiting 10 kDa)	458± 4.5	125±11	7.59±1.5*	40.8±4.0*
Filtrates (limiting 3 kDa)	243±1.9**	128±13	7.50±0.91*	26.5±2.2*
Shoma				
Original solution	1270±11	122± 8.5	5.34±0.88*	57.7±6.5*
Filtrates (limiting 10 kDa)	1220±12	123±10	5.11±0.72*	60.1±6.3*
Filtrates (limiting 3 kDa)	886± 7.5**	125±11	4.87±4.2*	25.9±2.1*
Bofu				
Original solution	291± 3.1	128±13	12.3 ±1.1*	22.2±1.6*
Filtrates (limiting 10 kDa)	285± 2.5	128±11	12.0 ±1.1*	21.9±1.5*
Filtrates (limiting 3 kDa)	157± 2.1**	128±13	12.5 ±1.2*	18.2±1.9*
Ryutan				
Original solution	289± 2.1	122± 8.5	11.3 ±0.87*	32.7±3.1*
Filtrates (limiting 10 kDa)	290± 4.5	125±11	10.9 ±0.90*	30.9±3.0*
Filtrates (limiting 3 kDa)	176± 2.0**	117± 9.2	11.3 ±0.75*	20.4±1.5*
Kanzo				
Original solution	654± 5.5	130± 5.0	6.75±0.55*	34.0±3.2*
Filtrates (limiting 10 kDa)	641± 5.0	126± 8.8	6.81±0.71*	35.0±3.1*
Filtrates (limiting 3 kDa)	329± 3.0**	127± 8.5	6.81±0.77*	20.3±2.1*

Starting concentrations of calcium and phosphate were 3 mM each. An aliquot of the original Rikko-san, its five herbal consitutents or their filtrates was added to the reaction mixture 5 min before the addition of 3 mM phosphate. The volume of all additives were 80 μl, and their concentrations were all adjusted to 10 mg/ml. The experimental conditions were the same as shown in Table I.

*Indicates a significant difference ($p<0.05$) when compared to the control.

**Indicates a significant difference ($p<0.05$) when compared to the polyphenol contents of the original solution.

tions were increased. A daily dose of 1.5 g of Rikko-san can be orally administered to a patient.[9] At this dose, more calcium chelation could take place. Therefore, in contrast to HEBP, Kampo medicines seem to exhibit inhibitory effects by two mechanisms; (i) the inhibition of crystal growth and (ii) the sequestration of calcium from the solutions. Further studies are needed to elucidate these points, because chelating agents could hurt the teeth.

The filtrates of Rikko-san through a membrane limiting the molecular weight of 10 kDa showed inhibitory effects on both the rate of HAP transformation and the induction time with a high polyphenol content (96 % recovery). Those of Rikko-san and five herbs through a membrane limiting 3 kDa showed a similar inhibitory effect on the rate of HAP transformation but their ability to extend the induction time was largely reduced (Table IV). This indicated that compounds with molecular weight below 3 kDa may inhibit the rate of HAP transformation in calcium phosphate precipitation, whereas compounds with a molecular weight between 3 and 10 kDa may be responsible for extending the induction time. Because such inhibitions on the rate of HAP transformation accompanied the 70 - 50 % recovery of polyphenol contents, polyphenols seem to be a causative substance for the decrease of the rate of HAP transformation. This situation is similar to the case of effective fraction from Shigyaku-san and Shikunshi-to.[14] Polyphenols are contained commonly in medicinal plants,[15] and those in beverages-especially teas, have been suggested to have anticariogenic activity.[16-18] This suggests that polyphenols may be important substances for oral hygiene in regard to plaque and calculus formations. The polyphenolic compounds seem to exert its preventive ability on the dental caries either by inhibiting glucosyltransferase activity[17] or binding to glycoprotein in saliva.[18] These components would also influence the formation of dental calculus and re-mineralization[19] by absorbing to the enamel surface of teeth and by directly inhibiting crystal growth of hydoxyapatite.

Traditional Chinese (Kampo) medicines which were developed over some 3,000 yrs[20] ago and have been designed to have low toxicity, may offer advantages over synthetic agents. Extraction of effective compounds from herbs or natural sources has resulted in many useful medicines.[21] We extracted a compound which had an inhibitory effect on the formation of calcium phosphate precipitates and found that the compound may be polyphenols.

In summary, we carried out fractionation of a Kampo medicine and its herbal constituents and studied their effects on the calcium phosphate precipitation. The effective fractions from Rikko-san and its five herbs were polyphenol with the molecular weight between 3 and 10 kDa for extending the induction time and those below 3 kDa for the rate of HAP transformation.

Acknowledgements

This work was supported by a research grant from Tsumura & Co., Japan.

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