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Purification and Properties of HPS (Halitosis Prevention Substance) Isolated from Cumin (*Cuminum cyminum L.*) Seed

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Abstract Halitosis is mainly caused by the presence of volatile sulfur-containing compounds (VSC's) produced by proteolytic periodontopathic bacteria in the oral cavity. Various mouth-rinses have been offered on the market as solutions to reduce halitosis. The aim of this study was to find a potent substance for the prevention of halitosis. The halitosis prevention substance (HPS) from cumin seed powder was purified by solvent extraction, silica gel column chromatography and preparative TLC to yield an oil phase (0.98%). Instrumental analysis such as FT-IR, ^{1}H-NMR and ^{13}C-NMR showed that HPS contained an -OH group, -HC=CH-, -COO-, and long chain acyl group. HPS was therefore determined to be 2-hydroxyethyl-β-undecenate. HPS inhibited the growth of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, by 72.44% and 64.37% at 1×10^{-2} M, and by 99.85% and 91.62% at 5×10^{-2} M, respectively. It also inhibited the activity of L-methionine-α-deamino-γ-mercaptomethane-lyase (METase), which was produced by oral microbes. Furthermore, the VSC production by oral microbes in the human mouth air decreased with increasing HPS concentration. These results suggested that HPS from cumin seed is an efficient halitosis prevention agent.

Keywords: Halitosis, 2-hydroxyethyl-β-undecenate, Cumin (*Cuminum cyminum* L.) seed

Introduction

Halitosis, commonly known as bad breath or oral malodor, arises from microbial degradation of proteins (especially those containing cysteine and methionine), peptides and amino acid that are present in the saliva, gingival cervical fluid or food retained on teeth (1). Halitosis is though to occur under anaerobic conditions, involving a range of proteolytic obligate anaerobes, especially the gram-negative species, such as *Fusobacterium* SPP, *Veillonella* SPP, *Treponema denticola, Porphyromonas gingival* and *Peptostreptococcus anaerobius,* that colonize the sub-gingival plaque and dorsum of the tongue (2, 3, 4).

Amongst these oral microorganisms, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* have been regarded as the predominate organisms associated with gingivitis and periodontitis and these organisms produced high levels of halitosis (5, 6, 7). The bacteria hydrolyze the proteins to amino acid, which contains sulfur functional groups and their precursors, and to volatile sulfur compounds (VSC's), which are gaseous substances that are responsible for halitosis and consist primarily of hydrogen sulfide, dimethyl sulfide, methylmercaptan and sulfur dioxide (6,8,9,10).

Amongst the VSC's, methylmercaptan has been regarded as the major contributor to halitosis. Methyl-mercaptan is transformed from L-methionine by L-Methionine-α-deamino-γ-mercaptomethane-lyase (METase). This enzyme catalyzes the α,β-or α,γ-elimination reaction of not only L-methionine, but also L-homocysteine, L-homocysteine, L-cysteine and S-adenosylmethionine (11, 12). Also, methylmercaptan can significantly reduce collagen content, DNA synthesis and proline transport in the oral cavity, and adversely affect the cellular integrity of intact oral mucosa (13). In seeking specific substrates for the prevention of halitosis from natural sources, we investigated cumin seed, which is known to be a food additive and Indian spice.

Cumin (*cuminun cyminum* Linn) is an annual plant of the family umbelliferae, and cumin seed is generally used as a food additive in the form of powder for imparting flavor to different food preparations. In addition, cumin has been used as a medicine since ancient times and has long been known to have antibacterial, antifungal and antiviral activities (14, 15). Accordingly, we investigated the potential for the purified potent substance for the prevention of halitosis from cumin seed. The purified substance was subjected to in vitro tests included antimicrobial plate assay, L-methionine-α-deamino-γ-mercapto methane-lyase inhibitory activity and a conductometric method to evaluate the level of methyl-mercaptan released by *Fusobacterium nucleatum* (ATCC 25586) and *Porphyromonas gingivalis* (ATCC 33277), which are the organisms known to be responsible for the production of VSC's in the oral cavity.

Materials and Methods

Materials Cumin seed powder was bought at Lotte market in Icsan, Korea. L-Methionine, L-Cysteine, DL-Homocysteine, L-Ethionine and S-methyl-L-Cysteine were purchased from Sigma Co. Tedlay™ (Poly(vinyl fluoride)) sampling bags with polypropylene valves were used for gas dilution analysis and also for the collection of real samples. The colorimetric reagent and all chemicals were of analytical grade.

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Purification of HPS (halitosis prevention substance) Cumin seed powder (100 g) was extracted for 3 hrs in flask with $CHCl_3$: CH_3OH (2:1) at 40°C. The solvent was filtered using a Whatman 42 filter (Whatman Int. Ltd. Maidstone. UK) and evaporated at 50°C. The resulting crude surfactant was dissolved in a small amount of $CHCl_3$: CH_3OH (2:1) and applied onto a silica gel 60 (Merck, Darmstadt, Germany) column (30 × 3 cm) that was pre-equilibrated with $CHCl_3$. The column was eluted at a flow rate of 50 ml/h with 4 ml of each fraction by subsequently increasing the polarity: $CHCl_3$, $CHCl_3$: CH_3OH (9 : 1, 4 : 1, and 2: 1) and CH_3OH. The four fractions (Fr.1~Fr.4) were obtained by elution. The Fr. 1 fraction with antimicrobial activity was pooled, and applied onto a silica gel 60 column (50 × 3 cm) and eluted with $CHCl_3$: CH_3OH : distilled water (60 : 40 : 4) at a flow rate of 50 ml/h with 4 ml of each fraction. The four fractions (Fr.1-1~ Fr.1-4) were obtained by elution. The Fr. 1-4 fraction with antimicrobial activity was pooled and dried at 50. Then, the Fr 1-4 obtained was spotted on preparative TLC (silica gel 60, Merck Co.) to be developed in a solvent system ($CHCl_3$: CH_3OH : water = (60 : 40 : 4) for six hours. The three fractions (Fr.1-4-1~ Fr.1-4-3) were obtained by elution. Then, the active fraction (Fr. 1-4-1) with antimicrobial activity of oil phase was pooled and dried at 50°C. The percentage yields of the active fractions Fr. 1, Fr. 1-4 and Fr. 1-4-1 based on fresh raw material were 3.7%, 1.26% and 0.98% respectively. The purification procedure is summarized in Fig. 1. The active fraction (Fr. 1-4-1) was named as HPS (halitosis prevention substance), and its purity was determined by two-dimensional TLC and HPLC. The two-dimensional TLC was prepared with the flowing procedure. First 1 g/ml of sample solution was spotted on a TLC plate (Silica gel 60, F254 Merck Co.) to be developed in the solvent system ($CHCl_3$: CH_3OH : D.W =40 : 10 : 1) for five hours. Then, the plate were sufficiently dried for two-dimensional analysis by using the solvent system ($CHCl_3$: CH_3OH : acetic acid : water =100 : 20 : 12 : 5) the plates were treated with I_2 vapor in order to develop the spots. The purity was determined by HPLC (Shimadzu UV 1240, Kyoto, Japan) with RI detector (RID-10A, Shimadzu). The separation was made with a Pinnacle II C18 column (USP L-1, Shimadzu) eluted isocratically using 10% (v/v) acetonitrile (CAN) in hexane (35°C, 2 ml/min).

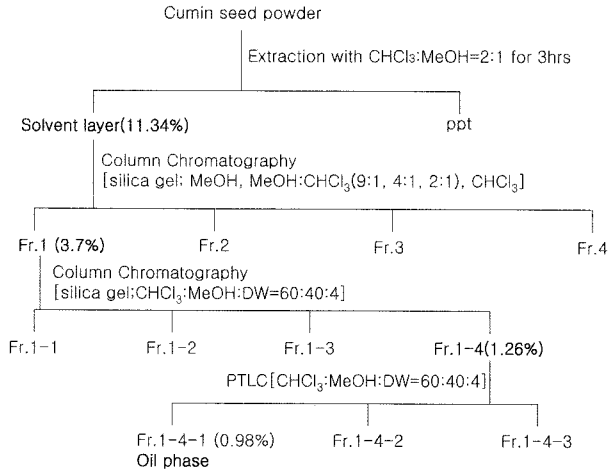

Fig. 1. Procedure for purification of HPS from cumin seed.

Interpretation of infrared spectrum The IR spectrum was obtained with a spectrophotometer (JASCO, IR-700, Japan), and the reagent was prepared according to the KBr purification method. KBr powder without reagent was pressure purified to be used as the reference substance, in order to determine the loss due to infrared scatter or absorption of impurity.

Interpretation of NMR (nuclear magnetic resonance) The NMR spectrum was measured using JEOL FX-100 NMR, based on the pulse Fourier transform method, with TMS [tetramethylsilane : $(CH_3)_4Si$] serving as the reference substance. Reagent (7~10 mg) was dissolved in $CDCl_3$ at the ratio of 5~20% (w/v).

Inhibitory effect of HPS on growth of *F. nucleatum* and *P. gingivalis* The organisms *Fusobacterium nucleatum* (ATCC 25586) and *Porphyromonas gingivalis* (ATCC 33277) were used as test organisms to investigate VSC production. The effect of HPS on the growth of the test organisms was determined after adding HPS at various concentrations from 5×10^{-4} M to 1×10^{-2} M to modified chopped meat (MCM) medium in a screw-cap tube. MCM medium was prepared according to KCCM strain catalogue (16). Then, the test strains were inoculated to the concentration of 4%(v/v) and cultured for 24 hrs in a CO_2 incubator at 37°C. The antimicrobial effect was spectrophotometrically measured by determining optical density (Varian DMS 200, USA) at 620 nm. One milliliter of this inoculum was added to each plate containing MCM agar to give final cell concentrations of 16^6~10^7 cfu/ml. Then HPS was added to 200 μl of 1×10^{-2} M inoculum in a 5-mm diameter, stainless cup cylinder, and water was added to provide a negative control. The cup cylinder was put in the middle of the plates which were incubated at 37°C for 24 hrs. Antimi-crobial activity was evaluated by measuring the zone of inhibition against the test organisms (17, 18).

Purification and inhibitory activity assay of METase from *F. nucleatum* and *P. gingivalis* The purification and inhibitory assay of METase from *F. nucleatum* and *P. gingivalis* were measured according to the method of Yosimura *et al.*(7).

F. nucleatum (ATCC 25586) and *P. gingivalis* (ATCC 33277) were grown anaerobically (10% CO_2, 10% H_2, 80 % N_2) in MCM broth at 37°C for 24 hrs and harvested by centrifugation. The bacteria pellet was suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA and 10 mM phenylmethylsulfonyl fluoride, and the cells were lysed by ultrasonification (Ultrasonic Processor GE 50, Sigma Co, St. Louis. Mo. USA).

The cell debris was removed by centrifugation at 10,000 × g for 20 min, and the supernatant was fractionated with ammonium sulfate. Protein precipitates between 50 and 70 % saturation with ammonium sulfate were collected by centrifugation at 15,000 × g for 30 min. The precipitate was

dissolved in 50 mM tris-HCl buffer (pH 7.5) containing 10 mM EDTA and 10 mM pyridoxal 5'-phosptate, and loaded on a 50 × 3 cm Sephadex G-100 gel filtration chromatography column equilibrated with the same buffer. The protein was eluted at 40ml/h with the same buffer, and the fraction corresponding to the peak of activity was desalted and concentrated with a 30 kDa cutout, centrifugal filter unit (Millipore Co.). The concentrated fraction was used as the crude METase and stored at -20°C.

METase inhibitory activity was assayed as described by Yoshimura *et al.*(13). Various amounts of HPS were mixed with 1 ml of METase (20 unit) which had been dissolved in 2 ml of 50 mM potassium phosphate buffer (pH 8.0), and pre-incubated at 37°C for 10 min. The pre-incubation solution was mixed with 0.5 ml of the same buffer containing 10 μm pyridoxal 5'-phosphate, 10 mM L-methionine. This substrate solution was added to start the reaction. After incubation for 10 min at 37°C, the reaction was terminated by adding 0.5 ml of 4.5% trichloroacetic acid. The suspension was centrifuged, and 0.5 ml of the supernatant was added to 0.5 ml of 0.05% 3-methyl-2-benzothiazolinone hydrazone in 1 ml of 1 M sodium acetate (pH 5.2) and then incubated at 50°C for 30 min. The amount of α-ketobutyrate produced was determined by spectrophotometry with A_{335}. One unit of METase activity was defined as the amount of enzyme releasing 1 μmole of α-ketobutyrate per hour. The relative inhibition rate was calculated using the following equation:

$$\text{Inhibition (\%)} = 1 - \frac{[\text{A(incubation)-A(control)}]}{[\text{A(enzyme)-A(blank)}]} \times 100$$

VSC assay of microbial culture air and human mouth air The assay of methylmercaptan (CH_3SH) and hydrogen sulfide (H_2S), which is liberated from the oral bacterial culture sample upon incubation at 37°C, contained in the mouth air of volunteers was performed with a modified version of the method described by Julio *et al.* (19, 20). *F. nucleatum* and *P. gingivalis* were grown anaerobically (10% CO_2, 10% H_2, 80% N_2) in MCM broth containing 1% L-methionine and from 5×10^{-4} M to 5×10^{-2} M of HPS at 37°C for 24 hrs, respectively, and air samples released during incubation were collected in a Tedlar™ bag. The mouth air sampling and analysis were carried out as described by Julio *et al.* (20). The subject group consisted of five volunteers. The volunteers first rinsed their mouths with 10 ml of various concentrations of HPS, after which the subjects were instructed to keep their mouths closed for 2 or 3 min and to refrain from talking in order to allow sufficient build-up of VSC. Then they slowly exhaled through a tube to collected 1 L of mouth air in a Tedlar™ bag. The air samples collected in these bags were introduced to the exponential dilution chamber, which was packed with neocuproine+Cu(II) methanolic solution.

The neocuproine+Cu(II) methanolic solutions were prepared by dissolving 0.055 g of neocuproine in 50 ml of methanol and adding 10 ml of 7.45×10^{-3} M $CuSO_4$. The air sample was reacted with neocuproine+Cu(II) for 10min at 20°C. The amount of VSC produced was determined by spectrophotometry at A_{470}. The amount of VSC produced was calibrated with the standard curve, be obtained by using 200 ppb (v/v) H_2S (date not shown).

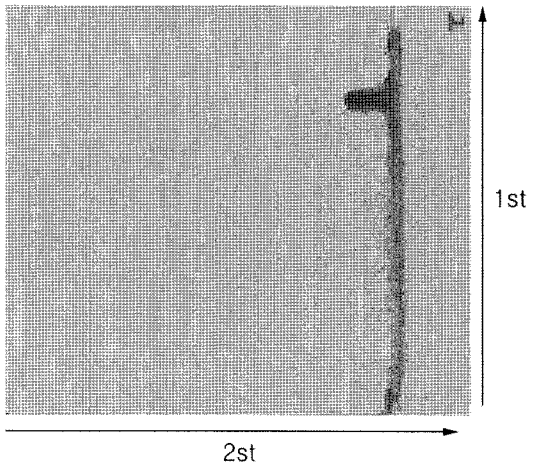

Fig. 2. Two dimensional thin layer chromatography of purified HPS. 1st: chloroform : methanol : 2M NH_3 = 40 : 10 : 1, 2nd: chloroform : methanol : acetic acid : water = 100 : 20 : 12 : 5.

Results and Discussion

Purification of HPS (halitosis prevention substance) HPS was purified from cumin seed powder by mixed solvent extraction, silica gel chromatography and preparative TLC as described in the Materials and Method. The final purification step produced an oil phase with an overall yield of 0.98%. The HPS purities by two-dimensional TLC and HPLC are shown in Figs. 2 and 3. HPS showed a single spot (R_f 0.79) in UV light and color reaction by iodine vapor after TLC (Fig. 2) and single band by HPLC, respectively (Fig. 3).

Interpretation of infrared spectrum In order to estimate the structural component of HPS, the absorption spectrum

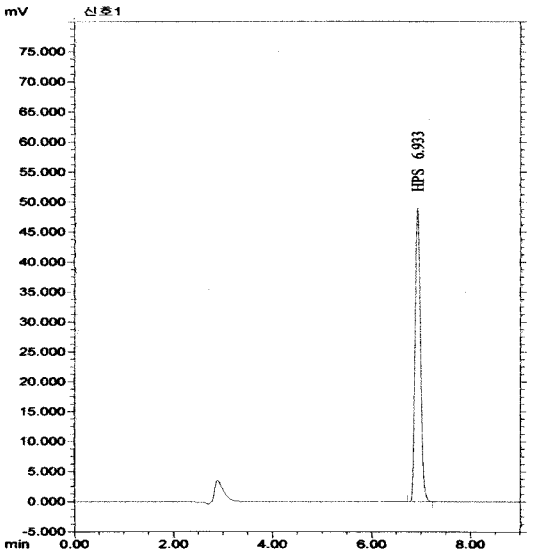

Fig. 3 HPLC spectrum of purified HPS.

was investigated by the KBr pellet method (Fig. 4). In the spectrum, the O-H group was shown at 3473 cm^{-1}, the specific absorption band of aliphatic CH_2-(C=O) at 2925 cm^{-1}, -CH_2- at 2854 cm^{-1}, -CH_2-CO-OR stretching band at 1745 cm^{-1} and 1169 cm^{-1}, and -CH_2OH stretching band at 1461 cm^{-1}.
Therefore HPS was determined to be an acyl compound with ester.

Interpretation of nuclear magnetic resonance (NMR) Figs. 5 and 6 show the ^{1}H-NMR and ^{13}C-NMR spectra of HPS purified from cumin seed powder. As seen in Fig. 5, the signals of the intense resonance at δ 0.79 and δ 1.18 were assigned to the methyl group (-CH_3) of the long chain (-C_7H_{14}-) and the regular chain of fatty acyl components, respectively. Also, -CH_2-CH= was shown at δ 1.60, =CH-CH_2- at 2.03 and -COO-CH_2- at δ 2.28. The small signals at δ 4.18, δ 4.25 and δ 5.32 were assigned to the -CH_2-CH_2-OH group. As seen in Fig. 6, ^{13}C-NMR spectrum for the isolated substance showed the ester carbonyl carbon at 173.2 ppm. In addition, the carbon signal of -HC=CH- was observed at 129.0 and 130.8 ppm, and the carbon signal of OH-CH_2-CH_2- at 62.1 and 68.9 ppm. The signals of 14.1, 22.7, 26.8, 27.3, 29.1, 29.4, 29.7 and 32.0 ppm corresponded to the methyl group and long chain hydrocarbon (-C_7H_{14}-CH_3). However, no branching signal was shown. It was concluded that the isolated compound that was purified in this study was an acyl compound containing a 2-hydroxy ethane and β-undecylenate. Therefore, HPS was determined to be 2-hydroxyethyl-β-undecenate, and this compound was considered to be a novel compound. The structure of the HPC purified in this study is given in Fig. 7.

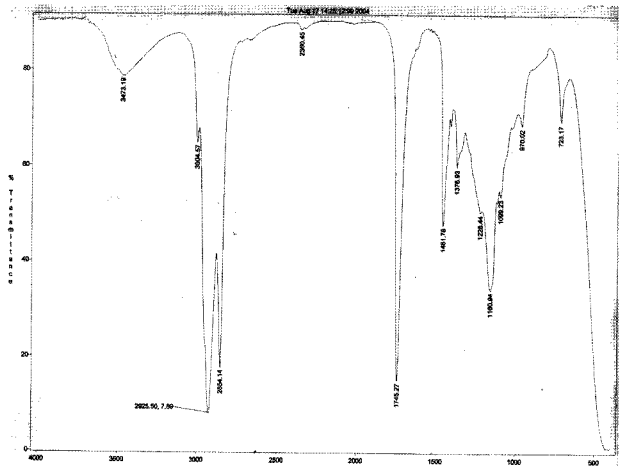

Fig. 4. FT-IR spectrum of HPS.

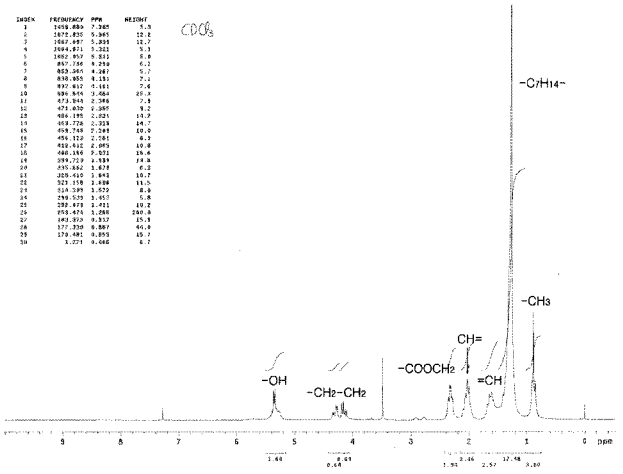

Fig. 5. ^{1}H-NMR spectrum of HPS.

Inhibitory effect of HPS on growth of *F. nucleatum* and *P. gingivalis* The fermentation characteristics of *F. nucleatum* and *P. gingivalis* with purified HPS were determined by measuring the growth of organisms after placing MCM broth into a screw-cap tube and HPS at a concentration ranging from 5×10^{-4} M to 1×10^{-2} M. As seen in Figs. 8 and 9, the lag phase was increased even at 1×10^{-2} M HPS, and the growth in the logarithmic phase was clearly delayed until 24 hrs of culturing. In addition, the inhibitory effect of HPS on the growth of *F. nucleatum* and *P. gingivalis* by agar plate method is shown in Fig. 10.

Inhibitory effect on METase of *F. nucleatum* and *P. gingivalis* The inhibitory effect on METase of HPS was determined by assaying the production of 2-ketobutyrate as described previously (21). The effect of HPS on the METase activity of *F. nucleatum* and *P. gingivalis* is shown in Table 1. The METase activity was inhibited by HPS and the amount of 2- ketobutyrate produced was decreased. The rate of inhibition was also increased with increasing concentration. In other words, the amount of 2-ketobutyrate produced from *F. nucleatum* METase was 0.426 ± 0.010 with 5×10^{-3} M HPS, 0.1888 ± 0.001 with 1×10^{-2} M and 0.001 ± 0.010 with 5×10^{-2} M of HPS. The rate of 2-ketobutyrate release was inhibited by 38.54%,

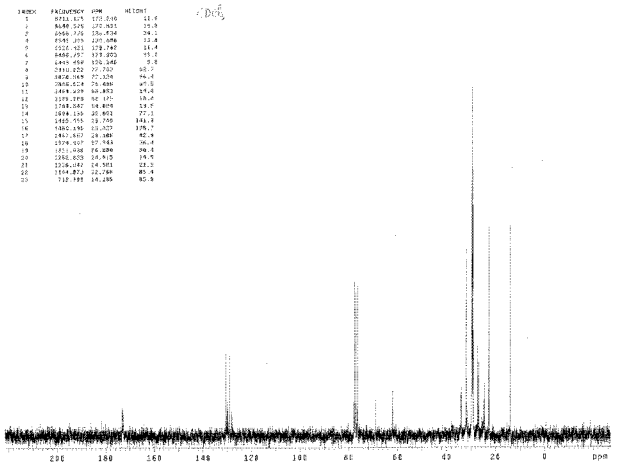

Fig. 6. ^{13}C-NMR spectrum of HPS.

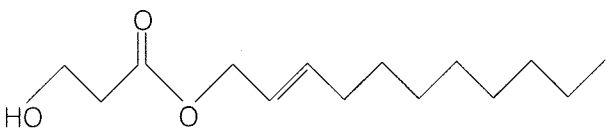

Fig. 7. HPS structure.

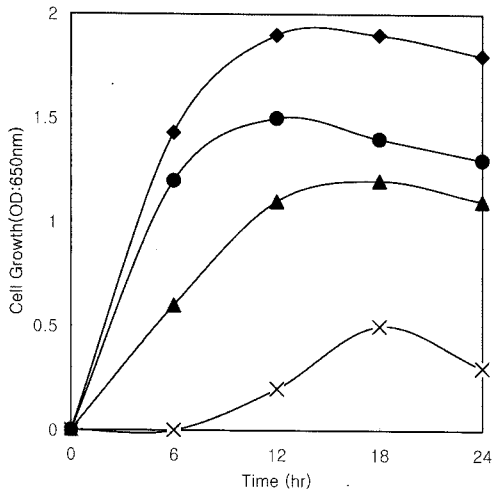

Fig. 8. Effect of HPS on the growth of *Fusobacterium nucleatum* (ATCC25586). ◆: Control ●: 5×10^{-4} M ▲: 2×10^{-3} M ×: 1×10^{-2} M.

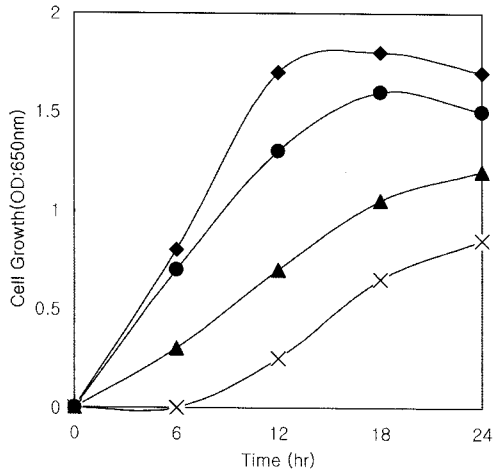

Fig. 9. Effect of HPS on the growth of *Porphyromonas gingivalis* (ATCC 33277) ◆: Control ●: 5×10^{-4} M ▲: 2×10^{-3} M × : 1×10^{-2} M.

72.44% and 99.85% with 5×10^{-3} M, 1×10^{-2} M and 5×10^{-2} M of HPS, respectively.

The absorbance of 2-ketobutyrate produced from *P. gingivalis* was 0.513 ± 0.021, 0.243 ± 0.001 and 0.064 ± 0.004 with 5×10^{-3} M, 1×10^{-2} M and 5×10^{-2} M of HPS, respectively, and the rate of 2-ketobutyrate release was inhibited by 24.79%, 64.37% and 91.62% with the same HPS concentrations, respectively. This enzyme catalyzes the α,γ-elimination of L-methionine to produced 2-ketobutyrate, methylmercaptan and ammonia. The methylmercaptan produced from L-methionine by the enzymatic action of METase is considered to be one of the main causes of halitosis (20, 21, 22).

Table 1. Inhibitory effect of HPS on L-methionine-α-deamino-γ-mercapto methane-lyase activity

Strain	Concentration (M)	Produced 2-ketobutyrate	Inhibition (%)
F. nucleatum (ATCC 25586)	0	0.682 ± 0.050	-
	1×10^{-3}	0.544 ± 0.010	21.24
	5×10^{-3}	0.426 ± 0.010	38.54
	1×10^{-2}	0.188 ± 0.001	72.44
	5×10^{-2}	0.001 ± 0.010	99.85
P. gingivalis (ATCC 33277)	1×10^{-3}	0.575 ± 0.012	16.69
	5×10^{-3}	0.513 ± 0.021	24.79
	1×10^{-2}	0.243 ± 0.001	64.37
	5×10^{-2}	0.064 ± 0.004	91.62

Yaegaki *et al.* (23) reported that the ratio of methyl mercaptan and hydrogen sulfide was greater in mouth air from patients with periodontal disease than in air from healthy men. These findings suggest that methyl mercaptan from periodontal pockets may be associated with the halitosis of patients with periodontitis. Persson *et al.* (24, 25) reported that the presence of 1 mM 3-chloro-DL-alanine had no effect on the growth of *P. gingivalis* and *F. nucleatum*, which produced a large amount of methyl mercaptan. In contrast, *Streptococcus mutans*, which does not produce methylmercaptan, was produced from L-methionine. Yoshimura *et al.* (13) reported that 3-chloro-DL-alanine inhibited the growth of METase-deficient mutants of *P. gingivalis*, and inhibited the METase activity in the presence of 1 mM 3-chloro-DL-alanine. Thus, HPS effectively inhibited METase activity even at low concentrations, and this antibacterial agent is therefore expected to be useful for specifically selecting halitosis bacteria producing large amounts of methylmercaptan from among the various oral bacteria. Furthermore, we hypothesize that this enzyme inhibition may be a

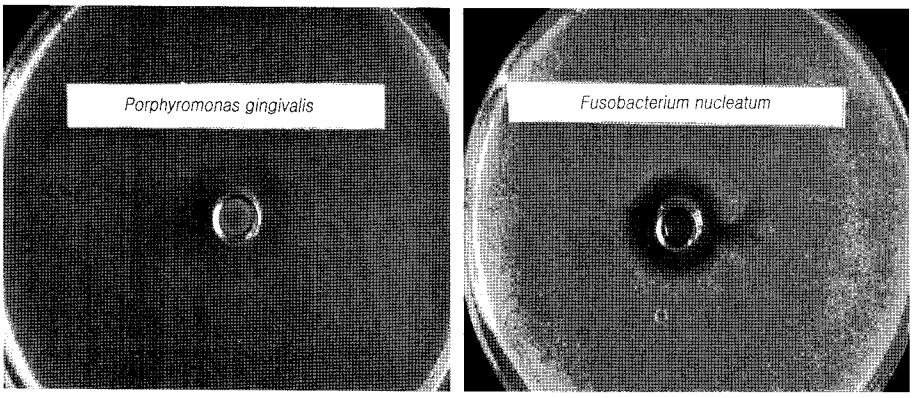

Fig. 10. Effect of HPS on the growth of *P. gingivalis* and *F. nucleatum* by diffusion method.

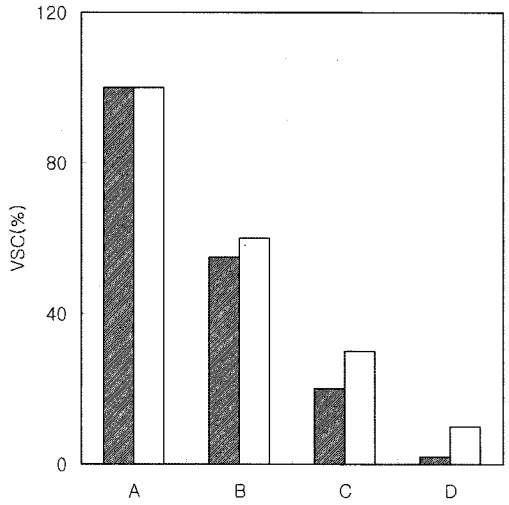

Fig. 11. Inhibitory effect of HPS on methylmercaptan and hydrogen sulfate (VSC) production from *F. nucleatum* and *P. gingivalis*. A: Control B: 5×10^{-3}M C: 1×10^{-2}M D: 5×10^{-2}M ▨ : *F. nucleatum* □ : *P. gingivalis*.

potentially exploitable target in the design of chemotherapeutic drugs with carcinogenicity, low-toxicity, and effective therapeutic application for humans.

VSC assay of microbial culture air and human mouth air The reaction of the neocuproine+Cu(II) with hydrogen sulfide and methylmercaptan is a redox process where a change in color is produced by the reduction of the complex by the sulfide ion (20). Neocuproine has been traditionally used with the aid of reducing agents for the determination of Cu (26). Neocuproine+Cu(II) reacts with sulfide ions producing a complex with a different color as a result of the reduction of the Cu(II) to Cu(I), and this reaction can be used for the determination of sulfide ion. When neocuproine+Cu (II) reacts with sulfide ion, the chelate is transformed into neocuproine-Cu(II), and changes its color from slight green to yellow (19). We are used this 'visual detection' at measurement of VSC. The effect of HPS on the production of VSC of *F. nucleatum* and *P. gingivalis* is shown in Fig. 11. The amount of VSC produced by oral microbes was decreased and this rate of decrease was increased with increasing concentration. The amount of VSC produced by *F. nucleatum* was 58.0, 22.0 and 3.6 ppb, and that of VSC produced by *P. gingivalis* was 64.0, 34.0 and 14.0 ppb, with the addition of 5×10^{-3} M, 1×10^{-2} M and 5×10^{-2} M HPS, respectively. The effect of HPS on VSC decrease in human mouth air is shown in Table 2. As the concentration of HPS increased, the amount of VSC produced was decreased. Cassino *et al.* (27) reported that zinc solution at 0.1% concentration decreased the VSC production in human mouth air by 95.68%. Thus HPS purified from cumin seed in this study inhibited VSC production even at low concentrations, unlike other substances previously reported in the literature.

Acknowledgments

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Table 2. Effect of HPS on VSC produced by human mouth air

Group	Volunteers	VSC (ppb)
	1	103.4 ± 6.2
	2	165.8 ± 9.9
0	3	167.3 ± 10.0
	4	97.8 ± 4.6
	5	124.6 ± 6.8
	6	75.7 ± 4.5
	7	86.3 ± 6.3
5×10^{-3} M	8	80.6 ± 4.8
	9	67.7 ± 4.1
	10	74.9 ± 3.8
	11	38.0 ± 2.5
	12	30.9 ± 2.2
1×10^{-2} M	13	34.1 ± 2.0
	14	28.0 ± 1.7
	15	40.2 ± 2.7
	16	11.9 ± 1.0
	17	21.5 ± 1.7
5×10^{-2} M	18	10.2 ± 0.8
	19	11.8 ± 0.9
	20	12.7 ± 1.0

References

1. Niranjan R, Nivedita R, Ritu I, Candasekaran S. Phenolic antibacterials from Piper betle in the prevention of halitosis. J. Ethnophamacol. 83: 149-152 (2002)
2. Kleinberg I, Codipilly M. The biological basis of oral malodor formation In: Rosenberg, Mel.(ed), Bad breath: research perspective, Ramot Publication, Tel Aviv University. pp. 13-39 (1995)
3. De Boever, E.H., Loesche W.T. The tongue microflora and tongue surface characteristics contribute to oral malodor. In: Van Steenberge, D. and Rosenberg, M(eds), Bad breadth : a multidisciplinary approach, Leuven Belgium: Leuven University press. pp. 111-121 (1996)
4. de Boever EH, Loesche WT. Assessing the contribution of anaerobic microflora of the tongue to oral malodor. JADA. 126: 1384-1393 (1995)
5. Tozetich J. Production and origin of oral malodor: are view of mechanism and method of analysis? J. Periodontal. 28: 13-20 (1993)
6. Yaegaki K, Sanada K. Biochemical and clinical factor influencing oral malodor in periodontal patient. J. Periodontal. 63: 783-789 (1992)
7. Yoshimura M, Yashoo N, Haruka F, Toshihiko K. 3-chloro-DL-alanine resistance by L-methionine-α-deamino-γ-mercaptomethane-lysis activity. FEBS Lett. 523: 119-122 (2002)
8. Kleinberg I, Westbay G. Salivary methanolic factors and involved in oral malodor formation. J. Periodontal. 63: 768-765 (1992)
9. Tonzetich J. Production and origin of oral malodor: a review of mechanism and methods of analysis. J. Periodontal. 28: 13-20 (1977)
10. Tessier J.F., Kulkarni G.V. Bad breadth: etiology and treatment. Periodontic oral health. Oct: 19-24 (1991)
11. Scully C, El-Maaythah M. Poter SR, Greenman J. Breadth odor: etiopathogenesis, assessment and management. Eur. J. Oral Sci. 105: 287-293 (1995)
12. Johnson PW, Ng W, Tonzetich J. Modification of human gingival fibroblast cell metabolism by methyl mercaptan. J, Periodontal. Res. 27: 476-483 (1992)
13. Yoshimura M, Nakamo Y, Yamashita Y, OHO T, saito T., Koga

T. Formation of methyl mercaptan from L-methionine by *Porphyromonas gingivalis*. Infet. Imun. 68: 6912-6916 (2000)
14. Singh KK, Goswami, TK. Mechanical properties of cumin seed (cuminum cyminum Linn.) under compressive loading. J. Food Eng. 36: 311-321 (1998)
15. Baxter R, Holzapfel W.H. Antimicrobial investigation of selected spices herbs, and additive in south Africa. J. Food Sci. 47: 570-578 (1982)
16. KCCM. catalogue of strain, 7th edition, KCCM press, p.421 (2001)
17. Lee HY, Jeong HS. Isolation and identification of antimicrobial substance from *Canavalis gladiate*. Food Sci. Biotechnol. 14: 268-274 (2005)
18. Ku JW, Huh JE, Kyung SH, Kyung KH. Antimicrobial effect alk(en)yl sulfides found in essential oil garlic and onion. Food Sci. Biotechnol. 13:235-239 (2004)
19. Julio RF, Reaina LF, Rosario P, Manuel M, Tose MT, Alberto S, Alfredo SM. A solvent tube for oral malodor monitoring. Talanta. 62: 421-426 (2004)
20. Julio RF, Jose MC, Rosario P, Alfredo SM. Simple detector for oral malodor based on spectrofluorometric measure-ments of hydrogen sulfide in mouth air. Anal. Chim. Acta 398: 23-31 (1999)
21. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning; a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp 100-124 (1989)
22. Tan Y, Xu M, Tan X, Wang X, Saikawa Y, Nagahama X, Lenz, M, Hoffman R.M. Overexpression and large-scale production of recombinant L-methionine-α-deamino-γ-mercapto methane-lyase for novel anticancer therapy. Protein Expr. Purif. 9: 233-245 (1997)
23. Yaegaki K, Sanada K. Biochemical and clinical factors influencing oral malodor in periodontal patients. J. Periodontal. 63:783-789 (1992)
24. Persson S, Edlund MB, Claesson, R, Carlsson J. The formation of hydrogen sulfide and methylmercaptan by oral bacteria. Oral Microbiol. Immunol. 5: 195-201 (1990)
25. Persson S, Claesson R, Carlsson J. The capacity of subgingival microbiotas to produce volatile sulfur compounds in human serum. Oral Microbiol. Immunol. 4: 169-172 (1989)
26. Sandell EB (ed). Colorimetric determination of trace of metal. Interscience. New York. 1959. p.187-188 (1990)
27. Cassiano KR, Grazyna J, Gunnar R. Comparative analysis of some mouthrinses on the production of volatile sulfur-containing compounds. Acta Odontol. Scand. 60: 10-12 (2002)