Foot odor due to microbial metabolism and its control

Katsutoshi Ara, Masakatsu Hama, Syunichi Akiba, Kenzo Koike, Koichi Okisaka, Toyoki Hagura, Tetsuro Kamiya, and Fusao Tomita

Abstract: To characterize foot odor, we analyzed its components by sensory tests, isolated microorganisms that produce it, and evaluated the mechanism of the occurrence of foot odor. As a result, foot odor was found to be derived from isovaleric acid, which is produced when *Staphylococcus epidermidis*, a resident species of the normal cutaneous microbial flora, degrades leucine present in sweat. In addition, *Bacillus subtilis* was detected in the plantar skin of subjects with strong foot odor, and this species was shown to be closely associated with increased foot odor. Therefore, we screened various naturally occurring substances and fragrant agents that inhibit microbial production of foot odor without disturbing the normal microbial flora of the human skin. As a result, we identified citral, citronellal, and geraniol as fragrant agents that inhibit the generation of isovaleric acid at low concentrations.

Key words: foot odor, microbial metabolism, isovaleric acid, leucine dehydrogenase.

Résumé : Afin d'expliquer l'odeur de pieds, nous avons analysé ses composantes par tests sensoriels, nous avons isolé et les micro-organismes qui le produisent et avons évalué le mécanisme d'apparition de l'odeur de pieds. De ces travaux, il fut déterminé que l'odeur de pieds était dérivée de l'acide isovalérique qui est produite lorsque *Staphylococcus epidermidis*, une bactérie indigène de la microflore cutanée normale, dégrade la leucine présente dans la sueur. De plus, *Bacillus subtilis* fut détecté dans la peau plantaire des sujets ayant une forte odeur de pieds et il fut démontré que cette espèce était fortement associée à l'augmentation de l'odeur de pieds. Par conséquent, nous avons criblé diverses substances naturelles et agents fragrants qui inhiberaient la production microbienne d'odeurs de pieds sans déranger la microflore normale de la peau humaine. Nous avons ainsi identifié le citral, le citronellal et le geraniol comme agents fragrants inhibant la génération d'acide isovalérique à basses concentrations.

Mots clés : odeurs de pieds, métabolisme microbien, acide isovalérique, leucine déshydogénase.

[Traduit par la Rédaction]

Introduction

Unpleasant odors of the human body include those emitted from the entire body, i.e., by sweat, sebaceous, and senile glands, and those, such as halitosis, hair odor, and foot odor, emitted from particular parts of the body. Thus, identification of the primary components in each body odor and clarification of the mechanisms of their generation are important for the development of methods to effectively control such odors. For example, hair odor has been reported to be caused by short-chain fatty acids and aldehydes produced when *Staphylococcus capitis* and *Pityrosporum ovale* (*Malassezia furfur*), which are components of the normal flora of the scalp with strong lipase activities, decompose sebum secreted from the scalp (Yamamoto et al. 1998). Marshall et al. (1988) examined the association between microflora found on normal feet and foot odor. They found that high population densities of staphylococci and aerobic coryneform bacteria were associated with foot odor. In a further communication, they screened for exo-enzymes (lipase, protease, and callous degrading enzymes) and all microorganisms from feet. Feet with strong odors had significantly higher population densities of microorganisms that produced these exo-enzymes than feet with weaker odors. Kanda et al. (1990) found that isomers of short-chain fatty acids were the primary components of foot odor, and Kobayashi (1990) found that *Staphylococcus epidermidis*, which is a normal resident of the skin, plays a major role in the development of foot odor. Hircus is also reportedly caused by volatile steroids generated when androstene derivatives contained in axillary secretions, such as sweat and secretions from apocrine sweat glands, are decomposed by coryneform bacteria, such as *Propionibacterium acnes* and *Brevibacterium*, which are normal residents of the axillary region (Rennie et al. 1990). Zeng et al. (1996) also

Received 31 May 2005. Revision received 11 October 2005. Accepted 19 October 2005. Published on the NRC Research Press Web site at http://cjm.nrc.ca on 11 April 2006.

K. Ara[1], M. Hama, and S. Akiba. Biological Science Laboratories, Kao Co., Ltd., 2606 Akabane, Ichikaimachi, Haga, Tochigi 321-3497, Japan.
K. Koike. Hair Care Products Research Laboratories, Kao Co., Ltd., 2-1-3 Bunka, Sumidaku, Tokyo 131-8501, Japan.
K. Okisaka. Health Care Products Research Laboratories, Kao Co., Ltd. 2-1-3 Bunka, Sumidaku, Tokyo 131-8501, Japan.
T. Hagura, and T. Kamiya. Skin Care Products Research Laboratories, Kao Co., Ltd., 2-1-3 Bunka, Sumidaku, Tokyo 131-8501, Japan.
F. Tomita. Hokkaido Study Center, The University of Air, Kita-17, Nishi-8, Kitaku, Sapporo 060-0817, Japan.

[1]Corresponding author (e-mail: ara.katsutoshi@kao.co.jp).

 doi:10.1139/W05-130

reported the involvement of carrier proteins, such as odorous binding proteins, which transport the odorous compound 3M2H to the skin surface. In addition, Gozu et al. (2000) identified nonenal, an unsaturated aldehyde generated by oxidative decomposition of hexadecenoic acid in sebum, as the cause of senile odor.

We targeted foot odor in this study to clarify the mechanism by which microorganisms produce unpleasant odors, to screen plant extracts and fragrant agents that inhibit the routes of production of these, and thus, to propose safe and effective deodorants.

Materials and methods

Screening for bacteria that cause foot odor

Bacteria attached to the surface of feet were sampled from 96 females aged 20–30 years by compressing various stamp media to feet. Furthermore, resident and semi-resident microorganisms attached tightly to the skin were collected by bringing a cylindrical glass cup (Φ15 mm, 1.8 cm^2) into tight contact with the sampling site of the subjects feet, adding 1 mL of 50 mmol/L phosphate buffer (pH 7.0) supplemented with 0.1% Tween 80, gently scratching the skin surface with a Teflon rod, and recovering the fluid (scrub method). The samples were smeared on a medium (mannitol–salt medium, Eiken Chemicals; soybean–casein digest (SCD) medium, Eiken Chemicals; PUK medium (1.5% trypticase, 0.5% yeast extract (BBL), 0.5% heart extract (Nissui), 1% glycerol, 0.2% NaCl, 0.2% K_2HPO_4, 0.03% l-cystein monohydrochloride, 0.005% sodium oleinate, and 0.002% bromocresol purple), Kishishita 1978; and tryptosoy agar medium, Eiken Chemicals, supplemented with 0.5% Tween 80 and 0.2% L-leucine for staphylococci) and cultured under aerobic and anaerobic conditions. The anaerobic conditions of the broth culture consisted of an incubation at 37 °C for 3–4 days in a Glove box (ANX-1; Hirasawa Co., Ltd.) that contained a mixture of 80% N_2, 10% CO_2, and 10% H_2. Bacteria that proliferated on the media were harvested and identified by a method as follows after one repetition of a single colony isolation (SCI).

After repeating the SCI, colonies that appeared on the agar plates were identified by routine methods according to the colonization pattern, Gram-staining properties, microbial morphology, and the results of various biochemical tests (API kit, Bio-Merieux, Marcy l'Etoile, France; ApiStaph, ApiCoryne, Api20A, Api20NE, Api20E). The isolated microbial strains were cultured at 37 °C for 5 days under aerobic and anaerobic conditions using SCD medium (Difco) supplemented with 0.2% leucine and 0.5% Tween 80 and propionic acid selective medium supplemented with 0.2% leucine and 0.5% Tween 80.

A sensory test of vapor of the culture medium was carried out with regard to isovaleric-acid-like odor by five odor testers using the 5-point scale of level 1, no odor; level 2, slight odor; level 3, mild odor; level 4, moderate odor; and level 5, strong odor.

Extraction of DNA and analysis of the 16S rDNA gene

The genomic DNA was extracted using the PrepMan™ Ultra Reagent (Applied Biosystems, Foster City, California). A domain of about 1500–1600 bp that contained the entire base sequence of the 16S rRNA gene was amplified by PCR using extracted genomic RNA as the template, as described previously (Hiraishi 1992; Shinoda and Kato 2000). Two universal primers, 9F and 1541R, were used in PCR to amplify the 16S rRNA coding region, which was sequenced, and a base sequence of the sample was obtained. Purification and cycle sequencing of the PCR products were carried out using a ABI Prism BigDye Terminator Kit (Applied Biosystems). A DNA thermal cycler Gene Amp PCR System 9600 (Applied Biosystems) and an ABI PRISM 3100 DNA Sequencer (Applied Biosystems) were used, and the fragments of base sequences obtained were assembled with an Auto Assembler 2.1 (Applied Biosystems). The base sequences of the 16S rDNA obtained were analyzed by a homology study. Using the 16S rDNA in the sample, a molecular phylogenetic tree was prepared by the neighbor-joining method using Micro Seq Microbial Identification System software v.1.4.1 (J. Felsenstain and the University of Washington, Seattle, Washington), and the sequence was aligned using the Microseq Bacteria Full Gene Library v.0001 (Applied Biosystems). The sequence data determined in this study have been assigned DNA Data Bank of Japan (DDBJ) accession Nos. AB177641–AB177644.

Methods for the measurement of leucine dehydrogenase activity, and search for bacteria with leucine dehydrogenase

Enzyme activity was evaluated using the isolated *Staphylococcus* sp. H780, *Bacillus* sp. H20, and *Propionibacterium* sp. H456, as well as standard strains, such as *Staphylococcus epidermidis* IFO12993 and *Staphylococcus aureus* IFO13276. Bacteria were cultured in SCD agar medium that contained 0.5% Tween 80 and 0.2% L-leucine (30 °C, 2 days), collected, and washed with 50 mmol/L phosphate buffer (pH 7.0). Cocci and bacilli were treated, respectively, with 50 units of lysostaphin and 0.2% lysozyme at 37 °C for 1 h, sonicated, and centrifuged. The supernatant obtained was assayed by the above method for leucine dehydrogenase activity of the bacteria.

For the measurement of leucine dehydrogenase, 300 μL of an undiluted or appropriately diluted sample (culture fluid, ion-exchanged water for the control) was added to a reaction mixture that contained 1.5 mL of 250 mmol/L glycine buffer (pH 10.5), 1.0 mL of 60 mmol/L L-leucine, 93 μL of 100 mmol/L NAD^+, and 107 μL of distilled water, and the reaction was started at 30 °C. After the end of the reaction, the absorbance was measured at 340 nm, and the enzyme activity was calculated from the quantity of the generated NADH by determining changes in the absorbance (ΔOD/mL) per minute. The leucine dehydrogenase activity was calculated from the change in the quantity of the substrate calculated from the molar absorption coefficient of NADH at 340 nm by defining the quantity of the enzyme needed to generate α-ketoisocaproate from 1.0 μmol L-leucine/min at 30 °C and pH 10.5 as 1 U. The protein content of the enzyme fluid used was measured using a protein assay kit (BioRad, Hercules, California), and the activity per unit quantity of protein was calculated.

Measurement of short-chain fatty acids

In a test tube with a screw cap, 5 mL of the reaction

Table 1. Analysis of the quantities of various fatty acids in sweat odor.

Fatty acid	Value (ppm)*	Quantity of fatty acid (%)†
Acetic acid	1	85.1
Propionic acid	0.03	7.7
Isobutyric acid	0.0032	1.9
Butyric acid	0.002	0.8
Isovaleric acid	0.0023	2.3
Valeric acid	0.0062	0.5
Caproic acid	0.03	0.4
Caprylic acid	3	0.1
Capric acid	10	1.4
Total	—	100.0

Note: Samples were collected from the plantae of 30 subjects with foot odor. After extraction with ether, the extracts were concentrated in an evaporator at a normal pressure and 40 °C, and 2 μL was analyzed by GC–MS. The analysis was performed with a DB-WAX column (60 mol/L × 0.25 mm, ϕ 0.25 μm) using He as the carrier gas at a flow rate of 15 mL/min. The temperature was maintained at 60 °C for 5 min and then increased from 60 °C to 215 °C at 4 °C/min.

*The air threshold means the lowest concentration at which the odor can be perceived (determined using standard materials).

†Relative quantities of fatty acids present in the sweat sample.

mixture (the culture fluid obtained by suspending a microbial isolate from the planta with a liquid medium prepared by adding leucine at 0.2% and Tween 80 at 0.5% to SCD medium or PUK medium and cultivating it for 5 days), 40 ppm of benzoic acid (internal standard), and 2 mL of a methanol solution of methanol complex of boron trifluoride were added, and the mixture was incubated at 80 °C for 1 h. After cooling in air, 1.5 mL of hexane was added, and the mixture was stirred and centrifuged. The hexane layer was collected, concentrated, and analyzed on a Hewlett-Packard model 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, California). The column was a DB-1 (J&W Scientific, Folsom, California), the flow rate of He gas was 1.8 mL/min, and the spirit ratio was 1:50. The temperature was maintained at 80 °C for 5 min, increased from 80 °C to 300 °C over 20 min, and maintained at 300 °C for 15 min.

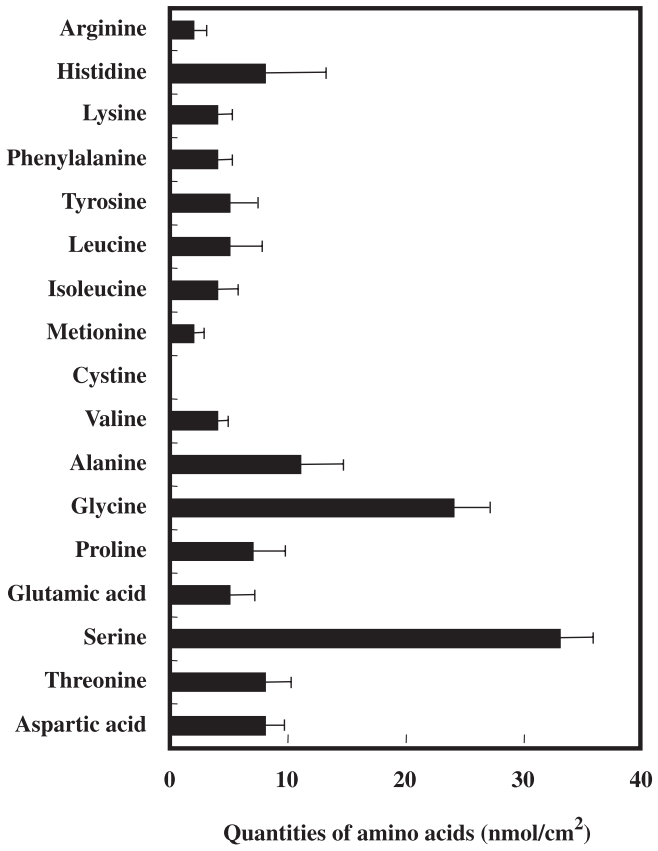
Analysis of amino acids in foot sweat

The samples collected by the scrub method (wash samples) were ultrafiltered using Centricon 10 (Amicon, Billerica, Massachusetts). Amino acids contained in the samples were analyzed by a fluorescent amino acid analyzer (L-8500, Hitachi, Hitachi-shi, Ibaraki, Japan) based on the *o*-phthalaldehyde method using a physiological amino acid column.

Search for agents that suppress the isovaleric acid production

The abilities of fragrant agents (extracts) to inhibit isovaleric acid production were evaluated by adding 30 μL each of the fragrant agents (5% ethanol solution) to a reaction mixture that contained 10 μL of leucine dehydrogenase derived from *Bacillus stearothermophilus* (Wako Chemicals) diluted to 2.5 U/mL with 100 mmol/L glycine buffer (pH 9.5), 0.75 mL of 250 mmol/L glycine buffer (pH 10.5), 0.5 mL of 60 mmol/L L-leucine, 49 μL of 100 mmol/L NAD^+, and 171 μL of distilled water. The absorbance was measured at 340 nm and 30 °C, and the decrease in the NADH production was compared with that of the control. The type of inhibition reaction was determined by plotting using the Edie–Hoftsee equation.

Fig. 1. Quantities of amino acids on the planta. Data are means of three independent experiments and bars indicate SD. Skin wash fluid per fixed area of the planta was collected by the scrub method from 10 healthy individuals, and, after ultrafiltration in Centricon 10, amino acid analysis was performed. Amino acid analysis was performed using L-8500, a fluorescent amino acid analyzer based on the *o*-phthalaldehyde method. The quantity of each amino acid is the mean per 1 cm^2 of the planta. The bars indicate standard deviations.

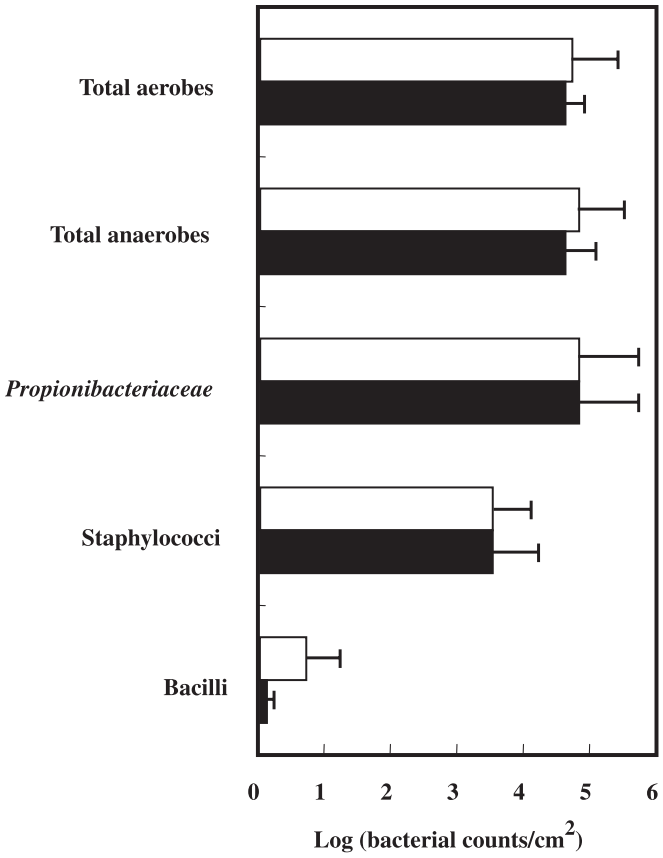


Results

Measurement of short-chain fatty acids

The quantity of each fatty acid in the sweat sampled from the feet was analyzed by GC, and percentages of various fatty acids were compared. Acetic acid, which causes the odor of sweat, accounted for 85.1% of the total amount of fatty acids dectected; isovaleric acid, which is responsible for foot odor, accounted for 2.3%, and propionic acid, isobutyric acid, and butyric acid, which are considered to contribute to foot odor, were also detected (Table 1). L-Leucine, which is considered to be involved in the generation of isovaleric acid, was detected at 2.5–7.5 nmol/cm^2 (Fig. 1).

Fig. 2. Relationship between the average number of bacteria and foot odor. (Open bar) Bacteria isolated from the plantae of subjects with foot odor judged to be level 3 or higher by a sensory test. (Solid bar) Bacteria isolated from the plantae of subjects with foot odor judged to be level 2 or lower by a sensory test. In 96 healthy individuals, the odor level of the planta was evaluated by a direct sensory test using a 5-point scale, and the subjects were divided into those at level 3 or above and those at level 2 or below. Plantar bacteria were collected using a direct stamp medium or scrubbing, and bacteria responsible for foot odor were isolated under aerobic and anaerobic conditions using various culture media containing leucine. The bacterial count is presented as the logarithm of the mean bacterial count per 1 cm^2. The bars show standard deviations.



Isolation of bacteria generating short-chain fatty acids

First, the microorganisms collected from 1 cm^2 of the plantar skin of the subjects with foot odor rated as level 3 or above (very strong) on a sensory test and from those with weaker foot odor were examined under aerobic and anaerobic conditions using the test media described in the Materials and methods section.

As shown in Fig. 2, no marked difference was observed in the total number of bacteria detected in test media for aerobic bacteria, anaerobic bacteria, *Propionibacteria*, or *Staphylococci*, but *Bacilli* were detected in larger numbers in the subjects with foot odor. Concerning the result of the isolation of various bacteria from the feet of healthy individuals using API kits, *Propionibacterium acnes* was detected in all subjects, and *Staphylococcus epidermidis* in 86.5%. *Micrococci* were present in 43.8% of subjects, *Staphylococcus aureus* in 7.3%, and *Bacillus subtilis* in 11.5%. As for bacteria per square centimetre, *Propionibacterium acnes* was detected in large numbers (5.8×10^4), followed by *Staphylococcus epidermidis* (3.2×10^3), micrococci (1.2×10^2), *Staphylococcus aureus*, and *Bacillus subtilis*. Furthermore, by screening bacterial strains isolated from the subjects with foot odor using API kits, 71 strains that could grow with L-leucine as the sole energy and carbon source were isolated, and 46 strains that generate an isovaleric-acid-like odor in liquid medium were obtained. These 71 strains were cultured in a liquid medium, and 46 strains that generated an isovaleric-acid-like odor were selected. Using API kits, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Corynebacterium minutissimum*, *Propionibacterium granulosum*, and *Bacillus subtilis* were detected as the major species in the skin bacterial flora of the subjects. The odor score of isovaleric acid was particularly notable in isolates of *Staphylococcus* sp. H780 (level 2), *Staphylococcus* sp. H604 (level 3), *Staphylococcus* sp. H292 (level 2), *Propionibacterium* sp. H832 (level 3.5), *Propionibacterium* sp. H835 (level 5), *Propionibacterium* sp. H456 (level 4), *Corynebacterium* sp. H996 (level 3), and *Bacillus* sp. H20 (level 5) on sensory testing.

As a result of analysis using MicroSeq (Applied Biosystems), the base sequence of the 16S rDNA of the isolated H780 was 99.9% analogous to that of *Staphylococcus epidermidis*. In the molecular phylogenetic tree, the 16S rDNA of the isolated H780 was shown to be a cluster of 16S rDNA of the genus *Staphylococcus*, and was included in the clusters formed by the 16S rDNA of *Staphylococcus epidermidis*. From the above results and the results of routine identification based on the colony morphology, staining properties, microbial morphology, and results of various biological tests of the isolates, the isolate H780 was estimated to be *Staphylococcus epidermidis*.

Similarly, the base sequence of the 16S rDNA of the isolated H20 showed the highest homology rate of 99.94% with the 16S rDNA of *Bacillus subtilis*, the 16S rDNA of the isolate H292 showed the highest homology rate of 99.6% with the 16S rDNA of *Staphylococcus hominis* subsp. *hominis*, and the 16S rDNA of the isolate H456 showed the highest homology rate of 99.14% with the 16S rDNA of *Propionibacterium avidum*. Therefore, the isolates H20, H292, and H456 were considered to be strains similar to *Bacillus subtilis*, *Staphylococcus hominis* subsp. *hominis*, and *Propionibacterium avidum*, respectively.

Evaluation of the leucine dehydrogenase activity of isolates

Leucine dehydrogenase activity was evaluated in H780, H604, H996, H832, H456, and H20, in which the odor of isovaleric acid was noticeable on sensory testing. It was highest in *Bacillus* sp. H20 at 510.3 ± 6.0 (U/g protein) and *Bacillus subtilis* JCM1465 at 483.6 ± 8.8 (U/g), followed by *Propionibacterium* sp. H456 (67.6 ± 0.9 U/g), *Propionibacterium* sp. H832 (56.3 ± 1.1 U/g), *Staphylococcus* sp. H780 (23.4 ± 2.6 U/g), *Staphylococcus* sp. H292 (19.2 ± 3.8 U/g), *Corynebacterium* sp. H996 (18.3 ± 3.5 U/g), and *Staphylococcus epidermidis* sp. IFO12993 (21.2 ± 1.4 U/g).

Table 2. Inhibitory effects of fragrant agents against isovaleric acid production.

Fragrant agent	Concn. (%)	Isovaleric acid production (ppm)	Inhibitory value (%)
Control	—	7.86	0
Citral	0.05	1.08	86.3
	0.1	0.83	89.4
Nerol	0.05	3.09	60.7
	0.1	2.10	73.3
Anethol	0.05	5.35	31.9
	0.1	5.17	34.2
Linalool	0.05	5.02	36.1
	0.1	3.50	55.5
Geraniol	0.05	3.14	60.0
	0.1	2.21	71.9
Limonene	0.05	3.47	55.9
	0.1	3.33	57.6

*Bacillus s*p. H20, *Staphylococcus* sp. H780, and *Corynebacterium* sp. H996 were cultured at 30 °C for 3 days in SCD medium containing 0.2% L-leucine, and the production of isovaleric acid was measured by GC. The isovaleric acid concentrations in the cultures were 46.6 ppm for *Bacillus* sp. H20, 3.4 ppm for *Propionibacterium* sp. H456, 3.1 ppm for *Propionibacterium* sp. H832, 5.3 ppm for *Staphylococcus* sp. H780, and 8.7 ppm for *Corynebacterium* sp. H996.

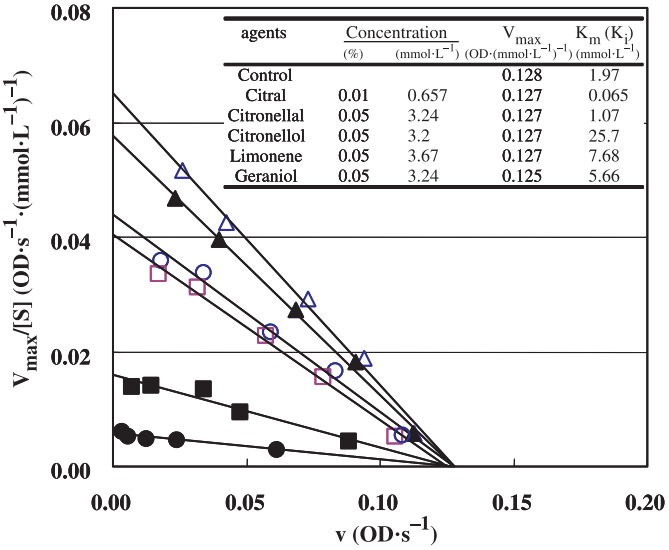
Search for plant extracts and fragrant agents that have leucine dehydrogenase inhibiting activity

About 60 naturally occurring fragrant agents were screened. High inhibition rates were observed in 0.05% and 0.1% each of citral, nerol, and geraniol (Table 2). Furthermore, the evaluation of the inhibition reactions of citral, citronellal, and geraniol showed that they were all competitive, and the K_i values were 0.065, 1.074, and 5.655 mmol/L, respectively, (Fig. 3).

Discussion

Amoore (1977) reported that the odor of isovaleric acid resembled human foot odor on the basis of the results of sensory testing, and Kanda et al. (1989) extracted materials from the socks and the plantar skin of patients with bromidrosis pedum and found that isovaleric acid was the key odor material. Because of this, we measured the quantities of various fatty acids in the sweat collected from the planta of healthy individuals by GC and calculated their percentages. Similar to the findings of Sawano (2000), acetic acid accounted for 85.1% of the total fatty acids collected, followed by propionic acid (7.7%), isovaleric acid (2.3%), and isobutyric acid (1.9%). However, since the thresholds of sensory detection of isovaleric and isobutyric acids are, respectively, 1/2000 and 1/3000 of that of acetic acid, isovaleric acid and isobutyric acid were shown to contribute greatly to foot odor.

Fig. 3. Inhibition of leucine dehydrogenase by addition of fragrant agents: ●, citral; ■, citronellal; □, citronellol; ○, limonene; and ▲, geraniol. △, Control. The straight lines were generated using the Edie–Hofstee equation. The values of the apparent inhibition constant K_i were calculated for each inhibitor concentration by using this equation.



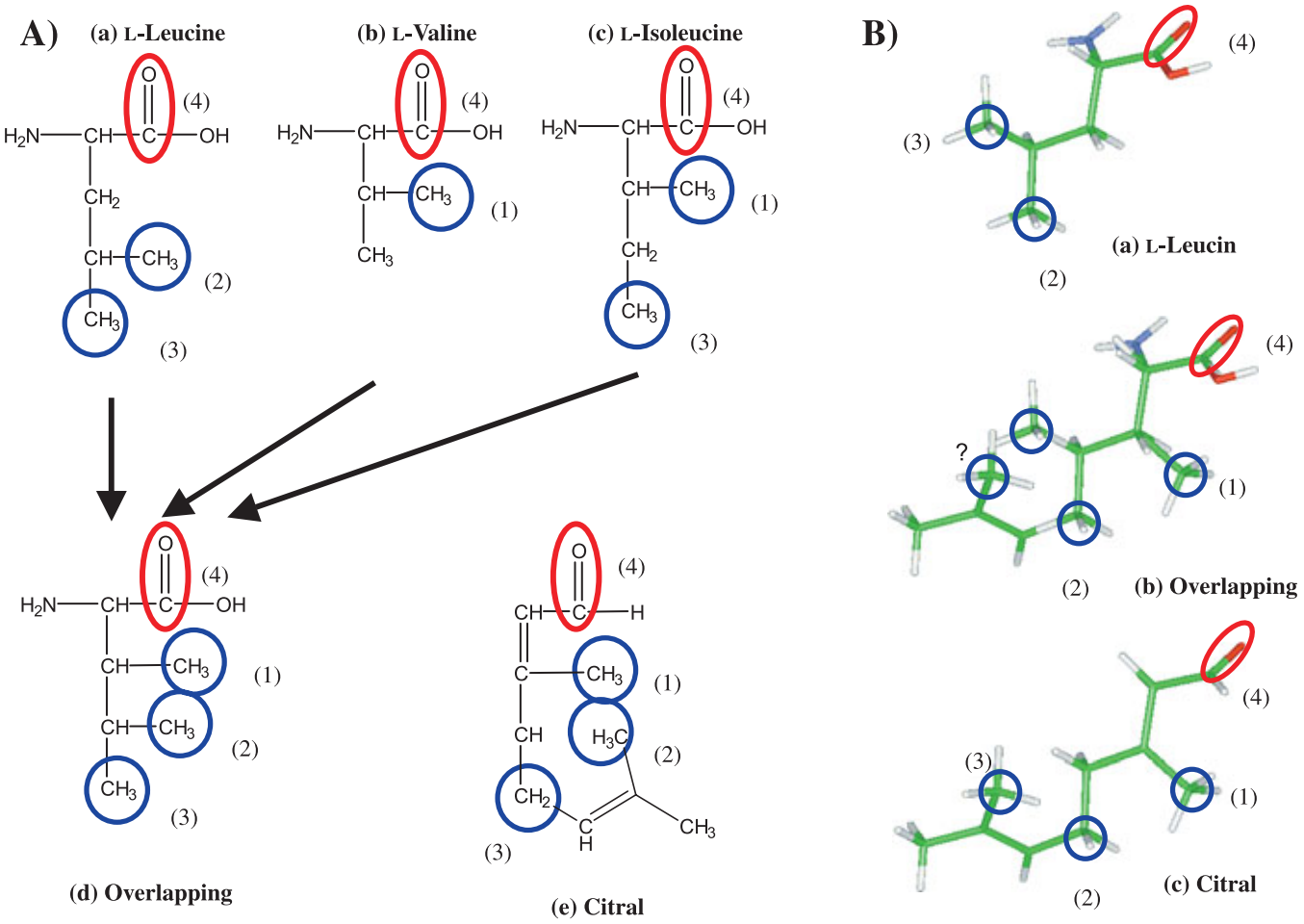
Similarly, we sampled eccrine sweat from the plantae of healthy individuals, allowed it to stand for a period with and without sterilization, and examined the generated volatile components by sensory tests. As a result, no increase in the odor was noted in the sterilized sweat, but the odor of lower fatty acids, particularly an isovaleric-acid-like odor, was increased in the nonsterilized sweat. We also measured the quantity of amino acids contained in sweat collected from the planta.

These analytical results indicate high contents of serine (about 30%) and alanine (about 10%), which are reported to be major constitutive amino acids of the natural moistening factor contained particularly in sweat. Also, acidic amino acid levels were slightly higher than basic amino acid levels. In addition, branched-chain amino acids, such as leucine, valine, and isoleucine, from which compounds responsible for foot odor (such as isovaleric acid and isobutyric acid) may be derived, were detected at similar levels.

The level of L-leucine was 2.5–7.5 nmol/cm^2; thus, leucine was shown to be present in the planta in a quantity sufficient to contribute to the generation of the odor. Branched-chain amino acids may be generated as scurf, or exfoliated corneal layers, are degraded by proteases of microorganisms inhabiting the planta, which are considered to supply amino acids for the generation of foot odor. From these observations, we speculated that foot odor is caused when organic compounds secreted from eccrine glands and sebaceous glands are degraded into volatile lower fatty acids, such as isovaleric acid, by microorganisms inhabiting the skin.

Moreover, to evaluate the relationship between components of the normal flora of the skin isolated from the foot and foot odor, we isolated and identified bacteria that generate foot odor from the planta using a medium containing leucine, and obtained 46 strains, including those of the genus *Staphylococcus*. When we inoculated a liquid medium

Fig. 4. Structural diagrams of leucine, valine, isoleucine, and citral (A) and diagrams of modeling (B), with overlapping of model molecules. Areas labelled 1–4 are the configurations considered to be commonly recognized by the enzyme. The red circles indicate the hydrophilic sites of recognition, and the blue circles indicate the hydrophobic sites of recognition.



containing leucine with these 46 strains and examined the volatile components generated immediately after the start of culture by sensory testing, acid odor and mild foot odor were perceived in the cultures of *Staphylococcus epidermidis*, *Staphylococcus hominis*, and *Corynebacterium minutissimum*; acid odor and intense foot odor were perceived in the cultures of *Staphylococcus aureus*, *Propionibacterium granulosum*, *Propionibacterium avidum*, and *Bacillus* spp.; and acid odor, intense foot odor, and indole odor were perceived in the culture of *Propionibacterium acnes*. Particularly, strains of the genus *Bacillus* were detected more frequently in the subjects with strong foot odor; they were considered to be involved in increases in foot odor.

Sawano (2000) reported that they perceived acid odor and sweat odor in the cultures of *Staphylococcus aureus*, an irritative strong acid odor in the cultures of *Corynebacterium minutissimum*, and acid odor, foot odor, and sweat odor in the cultures of *Brevibacterium epidermidis* and *Staphylococcus epidermidis*.

Takenaka et al. (2002) evaluated the metabolism of L-leucine to isovaleric acid by resident microorganisms of the skin (*Staphylococcus epidermidis* and *Staphylococcus aureus*) in vitro, and reported that, although isovaleric acid was produced by both species, it was particularly detected at 5 ppm or above in the cultures of *Staphylococcus epidermidis*.

We also observed the production of isovaleric acid at about 5–50 ppm by culturing bacteria of the genera *Bacillus*, *Corynebacterium*, and *Staphylocossus* in media containing L-leucine, the results of which support those of previous reports. Moreover, as a result of sensory tests of a standard material (isovaleric acid) by a panel, the odor of isovaleric acid could be perceived at concentrations of about 0.01 ppm or above. Therefore, the in vitro production of isovaleric acid is considered to be sufficient for the detection of the unpleasant odor.

These branched-chain fatty acids, including isovaleric acid, are expected to be generated by microorganisms from branched-chain amino acids, such as leucine and valine. Takemura et al. (2000) discovered that the rotten odor caused by *Bacillus subtilis natto* is accounted for by isobutyric acid, isovaleric acid, and 2-methylbutyric acid, and that this rotten odor disappears by inducing a gene defect of leucine dehydrogenase or branched-chain keto acid dehydrogenase, contained in *Bacillus subtilis natto*, which metabolizes leucine into isovaleric acid. Therefore, we speculated that isovaleric acid is produced on the skin when amino acids

present in sweat are metabolized by resident microorganisms of the skin, and measured enzyme activities of microorganisms inhabiting the skin. As a result, we observed that species of the genus *Bacillus* have very high leucine dehydrogenase activities.

According to Ohshima et al. (1978), leucine dehydrogenase is distributed in a very limited species, and it was detected in species of the genera *Bacillus*, *Clostridium*, *Thermoactinomyces*, *Corynebacterium* (*Corynebacterium sepedonicum*, *Corynebacterium pseudodiphtheriticum*), and *Alcaligenes* (*Alcaligenes faecalis*). They reported that the activity was particularly high in species of the genus *Bacillus*, with a spore-forming ability (0.30 U/mg protein in *Bacillus subtilis*, IFO3037; 0.18 U/mg protein in *Bacillus brevis,* IFO3331; 0.21 U/mg protein in *Bacillus licheniformis,* IFO12200), in agreement with our results.

Interestingly, we detected leucine dehydrogenase activities also in common skin residents, such as *Staphylococcus epidermidis*, and species of the genus *Propionibacterium*, *Corynebacterium* and *Bacteroides*. There have been reports that *Staphylococcus epidermidis* is involved in foot odor, and these reports are supported by the finding that this species produces leucine dehydrogenase.

According to the results of our study, species of the genus *Bacillus* were more often detected at high levels in individuals with strong foot odor, while resident bacterial species of the planta, such as staphylococci, are present at nearly fixed numbers. This finding suggests that species of the genus *Bacillus* greatly contribute to strong foot odor. As Marshall et al. (1988) also suggested, foot odor is considered to depend on the quantities of the enzymes that degrade the corneal layer and convert it to amino acids, and bacteria that possess enzymes that degrade such amino acids into odorous compounds or the presence of bacteria with very strong enzyme activities even in small quantities, such as those mentioned above, rather than the kinds of bacteria present on the planta.

Thus, we evaluated whether foot odor could be controlled by the inhibition of leucine dehydrogenase from bacteria that cause foot odor. It has also been reported that leucine dehydrogenase is competitively inhibited by D-form amino acids, and that it is markedly inhibited by D-leucine (inhibition rate 69%, K_i = 22 mmol/L).

We screened naturally occurring materials using leucine dehydrogenase activity as an index, and obtained citral (K_i = 0.065 mmol/L), citronellal (K_i = 1.074 mmol/L), and geraniol (K_i = 5.655 mmol/L), which were all competitive inhibitors. These results indicate that the above fragrant agents have greater affinities for leucine dehydrogenase than D-leucine, and inhibit the enzyme activity at low concentrations.

The leucine dehydrogenase that we used was derived from *Bacillus sphaericus*, and its substrate specificity relative to its activity to L-leucine (100%) was 74% for L-valine and 58% for L-isoleucine. Therefore, we extracted common characteristics (pharmacophores) of the four ligand molecules (leucine, valine, isoleucine, and citral) on the basis of the principles of ligand-based design (Fig. 4A) and identified sites 1–4 as possible sites recognized by this enzyme. When we then elucidated common characteristics of the three-dimensional structures of leucine and citral by overlapping them using modeling software (Insight 2; Accelrys Software Inc.), the structures of these compounds were found to nearly overlap at sites 1–4 (Fig. 4B). Thus, as sites 1–4 of citral enter the pocket of leucine dehydrogenase, the pocket becomes more hydrophobic and stable than when leucine enters the pocket. This may explain the higher affinity of the enzyme for citral than for leucine. Lacoste et al. (1996) reported that thymol and carvacrol, which are primary components of *Lippia sidoides* Cham., prevented *Corynebacterium xerosis* from causing hircismus but showed no specific inhibitory activity against the microbial flora of the planta. According to our results, thymol and carvacrol did not inhibit leucine dehydrogenase activity, and the results of analysis by molecular modeling suggest that they are not likely to prevent the generation of foot odor. Therefore, thymol and carvacrol are considered to specifically inhibit enzymes involved in the occurrence of hircismus. Thus, we demonstrated that leucine and other amino acids contained in sweat are decomposed on the skin by components of the normal flora with leucine dehydrogenase to produce isovaleric acid.

Particularly because *Staphylococcus epidermidis* and *Propionibacterium acnes*, which are major resident microorganisms of the human skin, produce isovaleric acid though in small quantities, they are considered to be responsible for the characteristic odor of the human body. Also, strong foot odor was found to be related to an increase in bacteria of the genus *Bacillus*, and they are considered to be a major factor in the quantity of isovaleric acid to an unpleasant level. We further demonstrated that leucine dehydrogenase inhibitors, such as citral, are effective for the control of foot odor.

Acknowledgements

The authors express their sincere gratitude to Mr. Tadahiro Ozawa, Bioscience Research Institute, Kao Corporation, for the modeling analysis concerning inhibitors of leucine dehydrogenase; Dr. Naonobu Yoshizuka, Bioscience Research Institute, Kao Corporation, for the valuable advice for the amino acid analysis; Mr. Satoru Hikichi and Mr. Hisato Itaya, Perfume Development Research Institute, Kao Corporation, for the sensory evaluations; and Mr. Atsushi Uzu and Mr. Akira Fuji, Skin Care Research Institute, Kao Corporation, for their valuable advice.

References

Amoore, J. 1977. Specific anosmia and the concept of primary odors. Chem. Senses Flavor, **2**: 267–281.

Gozu, Y., Haze, S., Nakamura, S., Kohno, Y., Fukui, H., Kumano, Y., Sawano, K., Ohta, H., and Yamazaki, K. 2000. Development of care-products to prevent aged body odor. J. Soc. Cosmet. Chem. Jpn. **34**: 379–386.

Hiraishi, A. 1992. Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. Lett. Appl. Microbiol. **15**: 210–213.

Kanda, F., Yagi, E., Fukuda, M., Nakajima, K., Ohta, T., Nakata, O., and Fujiyama, Y. 1989. Elucidating body malodor to develop a novel body odour quencher. J. Soc. Cosmet. Chem. Jap. **23**: 217–224.

Kanda, F., Yagi, E., Fukuda, M., Nakajima, K., Ohta, T., and Nakata, O. 1990. Elucidation of chemical compounds responsible for foot malodour. Br. J. Dermatol. **122**: 771–776.

Kishishita, M. 1978. The bacteriological and biological significance of *Propionibacterium acnes* — the indigenous bacteria of human skin. Acta Derm. **73**: 1–10.

Kobayashi, S. 1990. Relationship between an offensive smell given off from human foot and *Staphylococcus epidermidis*. Jpn. J. Bacteriol. **45**: 797–800.

Lacoste, E., Chaumont, J.P., Mandin, D., Plumel, M.M., and Matos, F.J. 1996. Antiseptic properties of essential oil of lippia sidoides cham. Application to the cutaneous microflora. Ann. Pharm. Fr. **54**: 228–230.

Marshall, J., Holland, K.T., and Gribbon, E.M. 1988. A comparative study of the cutaneous microflora of normal feet with low and high levels of odour. J. Appl. Bacteriol. **65**: 61–68.

Ohshima, T., Misano, H., and Soda, K. 1978. Properties of crystalline leucine dehydrogenase from *Bacillus sphaericus*. J. Biol. Chem. **253**: 5719–5725.

Rennie, P.J., Gower, D.B., Holland, K.T., Mallet, A.I., and Watkins, W.J. 1990. The skin microflora and the formation of human axillary odour. Int. J. Cosmet. Sci. **12**: 197–207.

Sawano, K. 2000. Body odors. Jpn. J. Taste Smell Res. **45**: 797–800.

Shinoda, Y., and Kato, N. 2000. Phylogenetic analysis of a bacterium by sequencing its 16S ribosomal RNA gene. Shimadzu. Rev. **57**: 121–132.

Takemura, K., Andou, N., and Tsukamoto, Y. 2000. Breeding of branched short-chain fatty acids non-producing natto bacteria and its application to production of natto with light smells. J. Jpn. Soc. Food Sci. Technol. **47**: 773–779.

Takenaka, H., Mikoshiba, S., Yokoyama, D., Hayashi, T., and Takada, K. 2002. Mechanism and regulation of body malodor generation. 51st Meeting of the Society of Cosmetic Chemists of Japan, 19 November 2002. pp. 68–71.

Yamamoto, H., Shimosato, I., and Okada, M. 1998. Study of fragrance materials on controlling head odors formation. J. Soc. Cosmet. Chem. Jpn. **32**: 26–32.

Zeng, C., Spelman, A.I., Vowels, B.R., Leyden, J.J., Biemann, K., and Preti, G. 1996. A human axillary odrant is carried by appolipoproteinD. Proc. Natl. Acad. Sci. U.S.A. **93**: 6626–6630.