Identification of Specific Odor Components in Mature Male Goat During the Breeding Season

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Abstract The present study aimed to identify a specific odor secreted by the mature male goat during the breeding season. Hairs from head and neck portions with the intensive odor were rinsed with chloroform-methanol (2:1, v/v) and washed by Folch's method. The chloroform layer (lipid fraction) was separated by cold acetone and the acetone soluble fraction was saponified. After methyl esterification of the saponified fraction, analyses with GLC, GC-MS, IR and NMR were done. Following extraction and fractionation, the odor was finally collected in the saponified fraction which contained free fatty acids. As a result of GLC analysis of the saponified fraction, over twenty peaks were obtained; distinct peaks which did not accord with standards were detected at about 4.5, 7.5, 13.5 and 23 min in retention time. Analyses of substances from these peaks with GC-MS, IR and NMR revealed the existence of 4-ethyl branched fatty acids. Biological tests on the reproductive behavior using synthetic 4-ethyloctanoic acid revealed that mature does including estrous ones were interested in the odor, suggesting that the odor of the mature male goat may have a role as a releaser pheromone.

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Many studies on odoriferous substances related to animal behavior have been done with insects, since BUTENANDT *et al.* first identified "Bombykol" from *Bombyx mori* as a pheromone in 1959¹⁾. In mammals, such study has been confined to laboratory or wild animals except for the pig²⁾.

We have been interested in the mature male goat, which secretes a specific odor during the breeding season. To clarify whether this odor acts as a sex pheromone, we have been studying its characteristics³⁾. In this report, we describe the components of the odor which were identified as ethyl-branched fatty acids.

Materials and Methods

From eight mature male goats of Saanen and Japanese Native breeds during the breeding season (September to November), hairs were cut from head and neck portions with intensive odor, and dorsum, nates and barba portions with no or slight odor.

The hairs were rinsed with several volumes of chloroform-methanol (2:1, v/v, C-M) by Folch's method⁴⁾ and the solvent was collected. The C-M extract was washed

with 0.2 volumes of 0.9% aqueous KCl and the lower layer (the chloroform layer) was collected. Fractionation was done by adding cold acetone and the fraction soluble in acetone was saponified with ethanolic KOH. As a result, the specific odor of all the bucks was collected in the saponified fraction including free fatty acids (Fig. 1).

For analysis, the saponified fraction was methyl-esterified with 5% anhydrous hydrogen chloride in methanol. GLC (Gas liquid chromatography, Shimazu, glass column 3 mm $\times$ 2.1 m, 1.5% OV-17 and 15% diethylene glycol succinate, FID), GLC for separation (JGC-110T, Jeol, stainless column 6 mm $\times$ 2 m, 20% Carbowax 20 M, CTD), GC-MS (M-52, Hitachi, glass column 2.5 mm $\times$ 1 m, 1.5% OV-17 or 3% SE 30, carrier gas He, ionized voltage 20 eV), IR (infra red, Jasco IRA-1), and ^1H - and ^{13}C -NMR (Jeol, JNM-FX-100, solvent CDCl_3 , tetramethylsilane for internal standard) were used for analysis.

Besides, a preliminary test on sexual behavior was done by using a synthetic compound. Fifteen to twenty does and two castrated bucks with non-detectable odor were used. In Observation I, a 40 cm long stick rolled with gauze soaked in the synthetic material or solvent was hung at 110–120 cm above the ground in a paddock (3^m $\times$ 10^m) of does. In Observation II, the one castrated buck was coated with the material and the other with solvent. These treated animals were placed by a fence of the paddock of does. In both observations, response behavior was recorded.

Results

By the extraction of hairs at head and neck portions of bucks with chloroform-methanol (2:1, v/v) and the subsequent fractionation with cold acetone and saponification, the specific odor was collected in the saponified fraction (the fraction of free fatty acids) (Fig. 1).

GLC of the methyl-esterified fraction of free fatty acids revealed four distinct peaks, which appeared at about 4.5 (peak 3), 7.5 (peak 6), 13.4 (peak 11) and 23 (peak 16) min in retention time and occupied 80~90% in total (Fig. 2). These peaks were not in accord with those of standard substances (methyl esterified saturated fatty acids) in which the relative retention time of $\text{C}_{16:0}$ was calculated as 1.00 (Table 1).

No difference was observed in the gas chromatogram between Saanen and Japanese Native breeds. On the other hand, GLC on the hair extracts from dorsum, nates, and barba portions in the buck in which the odor was barely recognizable resulted in only two peaks (6 and 11) of the above four being slightly detected. During the non-breeding season, these peaks almost vanished. In the specimens from immature males and females of various ages, these peaks were not detected even during the breeding season.

Analyses of the peaks 3, 6, 11 and 16 in GC-MS showed the same pattern on mass spectra in which the standard peak appeared at m/z 87 (Fig. 3). By separation with GLC, two peaks (peaks 6 and 11) were trapped and subjected to NMR analysis. In ^1H -NMR both peaks had the same pattern (signals were detected at $\delta_{\text{TMS}}^{\text{CDCl}_3}$ 0.85,

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Fig. 1. Extraction and fractionation of mature male goat odor

Fig. 2. GLC chromatogram of the methyl esters of the saponified fraction in which specific odor was detected.

Table 1. Relative retention time of peaks in Fig. 1.

Peak no.	Relative retention time*	Peak no.	Relative retention time*
1	0.067	11	0.4
2	0.096	12	0.484
C _{8:0}	0.097	C _{14:0}	0.524
3	0.137	13	0.543
4	0.151	14	0.661
C _{10:0}	0.153	15	0.679
5	0.184	16	0.739
6	0.224	17	0.984
7	0.270	C _{16:0}	1
C _{12:0}	0.283	18	1.098
8	0.295	19	1.242
9	0.317	20	1.415
10	0.351		

*As retention time of C_{16:0} is 1.00.

Fig. 3. Mass spectra of peaks 3, 6, 11, and 16 with GC-MS

0.88, 1.26, 1.60, 2.30 and 3.66 ppm), but only the intensity of the signal at 1.26 ppm was different, suggesting that both peaks contained $-\text{CH}_3$, $-\text{CH}_2$, $-\dot{\text{C}}\text{H}$ and $-\text{OCH}_3$ groups (Fig. 4).

Fig. 4. ^1H -NMR spectrum of peak 11 separated by GLC

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Fig. 5. ¹³C-NMR spectra of peak 11 separated by GLC

In the ¹³C-NMR analysis, the signal numbers of peaks 6 and 11 were thirteen and fifteen, respectively, and the signal pattern was very similar between them (Fig. 5). Thus, in peak 6 the signals were detected at 10.8, 14.2, 22.8, 25.6, 26.6, 28.3, 29.8, 31.7, 31.9, 32.9, 38.5, 51.5 and 174.6 ppm, while in peak 11, at 10.8, 14.2, 22.8, 25.6, 26.6, 28.3, 29.4, 29.8, 30.1, 31.7, 31.9, 32.9, 38.5, 51.5 and 174.2 ppm. These figures suggested that the structures of peaks 6 and 11 contained groups of C-C=O, -OCH₃, -CH, -CH₃ and -CH₂- for the signal(s) at 174.6, 51.5, 38.5, 14.2 and 10.8 ppm, and the others in ¹³C-NMR, respectively.

The chemical shift of α-carbon of esters was ascertained by selective decoupling in ¹³C-NMR measurement, that is, irradiation of α-hydrogen at 2.3 ppm in ¹H-NMR, resulted in a observation of a signal at 31.7 ppm as singlet. Therefore, it was considered that this signal originated from α-carbon. Two signals originated from the group of -CH₃ were detected at 14.2 and 10.8 ppm. In general⁶⁾, the terminal methyl group of fatty acid methyl esters is detected at 14 ppm. However, the signal at 10.8 ppm was higher, suggesting that branched-chain structure existed at the third carbon numbered from this methyl group (at 10.8 ppm) and that the methyl group function originated from the ethyl group. Also, the chemical shift of α-carbon detected at 31.7 ppm was shifted higher than that of α-carbon in saturated fatty acid esters usually detected at 34 ppm, suggesting that the branched-chain structure was at the third carbon counted from α-carbon. Consequently, it was concluded that peaks 6 and 11 were methyl esters of 4-ethyl decanoic and 4-ethyl dodecanoic acids, respectively. This conclusion was sustained by results of analyses with GC-MS and IR.

Peaks 3 and 16 showed the same pattern as peaks 6 and 11 in GC-MS, indicating that the formers were also the same branched-chain fatty acid methyl esters, although those peaks could not be analyzed with NMR and IR.

On the basis of this information, fatty acids with the estimated structures were synthesized⁶⁾ and the synthetic and natural substances completely accorded with respects to GLC, GC-MS, IR and NMR analyses. Thus, the components of the specific odor of the mature male goat during the breeding season were identified as 4-ethyl-branched fatty acids, i.e., 4-ethyl-octanoic, -decanoic, -dodecanoic, and -tetradecanoic acids.

Of the components of the specific odor identified in this study, a preliminary test was done by using synthetic 4-ethyloctanoic acid, the odor of which was most like the natural ones. In Observation I, five to six does including estrous ones approached the stick of material and sniffed it, while to the stick of solvent these animals did not react. In Observation II, estrous does were interested in the castrated buck coated with the synthetic material.

Discussion

In this study, substances belonging to the branched-chain fatty acids were identified as the components of the specific odor of the mature male goat during the breeding season.

The similar fatty acids have been known to occur in tissues and fluids of several animals⁷⁻¹¹⁾, most of which have a methyl branch. Lipids of hairs which are thought to originate from sebaceous glands contain iso- and anteiso-methyl-branched-fatty acids¹²⁾. Accordingly, they are mainly methylbranched, whereas there are few ethyl branched ones, as detected in the preen glands of Ciconiiform birds¹³⁾. As to the latter compounds, 4-ethyloctanoic acid has been reported as one of the components of perfume¹⁴⁾. Although JENKINSON *et al.*¹⁵⁾ have suggested that the components of the specific male goat odor are present in volatile fraction containing fatty acids of carbon number between C₁₀ and C₁₂, we first identified substances belonging to fatty acids.

WONG *et al.*¹⁶⁾ have isolated the components of volatile substances in sheep meat and have identified 4-methyloctanoic acid. They have reported that this substance is also included in goat meat, so that this odor is a common odor in both sheep and goat, named "goaty odor". The specific odor demonstrated in this study was, however, extracted and isolated from hairs at portions of head to neck of all the bucks in the breeding season, and related to the development of sebaceous glands⁹⁾. This odor is specific in the mature male goat, and does not occur apparently in immature animals or the female goat (unpublished data). Therefore, the goaty odor named by WONG *et al.* which occurs in goat and sheep meat seems to be different from the specific odor described in the present paper.

Effects of some odoriferous substances on the reproductive physiology have been

reported¹⁷⁾. Their actions are categorized into the direct effect on reproductive behavior (releaser pheromone) and into the indirect one on endocrine status mediated by hormonal systems (primer pheromone).

In sheep¹⁸⁾ with agnoistic treatment, noise played a valuable role in the ram's searching behavior to the female. However, in tests using odors alone, strict response has not been obtained so far in farm animals¹⁸⁾, although a study on the sexual behavior in the pig using a T maze has demonstrated that the estrous female responds to boar odor and an organic synthesized substance, 16-unsaturated C₁₉ steroid.

SHELTON and MORROW¹⁹⁾ and OTT *et al.*²⁰⁾ have recently found in the goat that existence of the mature buck stimulates the endocrine status of the doe, but the odor alone has little effect. KNIGHT and LYNCH²¹⁾ have reported that the introduction of a mature ram just before the breeding season stimulates and synchronizes ovulation in the ewe. We have observed⁹⁾ that an estrous doe has an interest in a mature buck with the intensive odor, but does not after the odor is shampooed away. In a preliminary test, mature does including estrous ones were interested in a castrated buck coated with synthetic 4-ethyloctanoic acid. These findings suggest that the specific odor in the male goat identified in this study may have a role as a releaser pheromone.

The mechanism of hormonal control and the organ producing the specific odor are still uncertain. Also, the pathway of biosynthesis must be studied in future.

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繁殖季節中の成熟雄ヤギ特異臭成分の同定について

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繁殖季節中に強く出現する成熟雄ヤギの特異臭について、成分の単離・同定を試みた。繁殖季節中に成熟雄ヤギ数頭から、特異臭が強く感知される頭一頸部の毛を刈り取り、クロロホルム-メタノール (2:1, v/v) で抽出した。続いて、クロロホルム層を冷アセトンで分画し、さらにアセトン可溶部をケン化した。ケン化画分をメチルエステル化後、GLC, GC-MS, IR および NMR で分析した。

特異臭は抽出・分画操作で最終的にケン化画分に集まった。GLC の結果で、20 以上のピークが得られたが、顕著なピークは保持時間約 4.5, 7.5, 13.5 および 23 分

に検出された。各種の機器分析で、これらの4つのピークは4位にエチル基のついた分枝脂肪酸、4-エチルオクタン酸、4-エチルデカン酸、4-エチルドデカン酸および4-エチルテトラデカン酸と同定された。有機合成を行い比較した結果、合成品と天然物は完全に一致した。合成品のうち、最も特異臭に近いにおいを持った4-エチルオクタン酸を用いた生物試験で、発情ヤギを含む成熟雌ヤギが興味を示し、成熟雄ヤギの特異臭がリリースファエロモンとして作用する可能性が示唆された。

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