Composition and Molecular Species of Waxy Lipids in Wheat Grain

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ABSTRACT

Waxy lipids such as acylsterols, shorter alkylesters, hydrocarbons, and longer alkylesters were isolated from wheat grain, and their components and molecular species were studied. The contents of these waxy lipid classes in neutral lipids were 7.4, 0.5, 0.4, and <0.1%, respectively. The major species of acylsterols were palmitoyl sitosterol and linoleoyl sitosterol; those of shorter alkylesters were methyl linoleate and methyl palmitate. Hydrocarbons, alkanes, alkenes, and squalene were detected, with alkanes being predominant. The main species of alkanes were C_{25}, C_{27}, and C_{29}, and those of alkenes were C_{25}, C_{29}, and C_{31}. The principal carbon numbers of longer alkylesters were C_{40}, C_{42}, C_{44}, and C_{46}, and the major species were palmitoyl hexacosanol, arachidoyl tetracosanol, palmitoyl tetracosanol, and behenoyl tetracosanol.

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Many studies on wheat lipids have been reported in the literature (Morrison 1978). We also have systematically studied triacylglycerols (triglycerides) and phospholipids (Ito et al 1984), glycosylglycerides (Ito et al 1983), and sphingolipids (Fujino and Ohnishi 1983) in wheat grain. However, studies on waxy lipids in wheat grain have not yet been carried out in detail, although there are a few reports about hydrocarbons (Kuksis 1964, Youngs and Gilles 1970, Lorenz and Maga 1975) and acylsterols (sterylesters) (MacMurray and Morrison 1970, Hsieh et al 1981, Laignelet 1983). This paper describes isolation, composition, and molecular species of the waxy lipids in wheat grain.

MATERIALS AND METHODS

Extraction and Fractionation of Total Lipids

Wheat grain (5 kg, Horoshiri variety) harvested at Hokkaido, Japan, in 1980 was ground to powder (30 mesh), steamed for 3 min to inactivate enzymes, and extracted by shaking with four volumes of a chloroform-methanol solution (2:1, v/v) three times and twice with three volumes of water-saturated butanol. The combined extracts were washed with water (Folch et al 1957), and the chloroform layer was evaporated to dryness to yield total lipids (2.7%). Part of the lipids (25 g) was applied to a silicic acid column (Rouser et al 1968) for fractionating into neutral lipids, glycolipids, and phospholipids. The ratio of each fraction obtained from the total lipids was 58:22:20.

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Isolation of Waxy Lipids

Part of the neutral lipid fraction (14 g) was analyzed on a 500-g Florisil column containing 7% water (Carrol 1961) to prepare waxy lipids. Hydrocarbons were eluted with 1,000 ml of hexane, acylsterols and longer alkylesters with 3,200 ml of hexane/diethyl ether (95:5, v/v), and shorter alkylesters with 4,000 ml of hexane/diethyl ether (85:15, v/v). Squalene, alkanes, and alkenes were separated by preparative silica gel G thin-layer chromatography (TLC) with hexane/benzene (19:1, v/v). Alkanes and alkenes were further isolated by silica gel G-$AgNO_3$ (95:5, w/w) TLC with hexane.

Longer alkylesters could not be separated well from acylsterols by the method reported previously (Ito et al 1981), because the R_f value of longer alkylesters was the same as that of acylsterols on silica gel G TLC. In addition, the amount of longer alkylesters was very small in wheat grain. Therefore, surface lipids were extracted from wheat grain, because longer alkylesters are located in cuticle tissues (Kolattukudy 1980). Thus, whole wheat kernels (2 kg) were dipped in hexane for 30 sec (Kolattukudy 1980), and the extracts were evaporated to dryness to obtain surface lipids (yield of 140 mg) from which a crude fraction of longer alkylesters was separated by silica gel G TLC with hexane/benzene (1:1, v/v), yielding 13% of the surface lipids.

Free sterols were also isolated from wheat grain by a combination of Florisil column chromatography and silica gel G TLC with hexane/diethyl ether (80:30, v/v) (Kuroda et al 1977).

Hydrolysis of Acylsterols and Longer Alkylesters

Acylsterols and longer alkylesters were saponified with 1*N* KOH in ethanol for 2 hr. Sterols and alcohols were extracted from the saponified mixture with diethyl ether. Fatty acids were then recovered with petroleum ether after the residual solution was acidified with 6*N* HCl. Alcohols and free fatty acids were analyzed as acetates (Kolattukudy 1970), trimethylsilyl ethers (Walton and

Kolattukudy 1972), and methylesters by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Sterols were also analyzed by GC and GC-MS.

Infrared Spectrometry

Infrared (IR) spectra were recorded on liquid films for squalene and shorter alkylesters, and in KBr pellets for alkanes, alkenes, acylsterols, and longer alkylesters, using an A-3 IR spectrophotometer (Japan Spectroscopic Co. Ltd., Tokyo).

GC

GC was carried out with a 163 gas chromatograph (Hitachi Seisakusho Co. Ltd., Tokyo) equipped with a hydrogen-flame ionization detector. Glass columns, 0.3 × 200 cm or 0.3 × 100 cm, were used with N_2 as the carrier gas. The column was packed with 1.5% OV-17 on Chromosorb WAW-DMCS for analysis of sterols, and the column temperature was held at 250°C. Fatty acid methylesters were chromatographed using a column packed with 5% diethyleneglycolsuccinate polyester on Chromosorb WAW-DMCS and temperature programmed from 175 to 195°C at a rate of 2°C/min. Alkanes, alkenes, and trimethylsilyl ether and acetyl derivatives of alcohols were analyzed on a column packed with 1.5% SE-30 on Chromosorb WAW-DMCS. The GC was programmed from 180 to 290°C at a rate of 2° C/min for separation of alkanes and alkenes and from 210 to 300°C at a rate of 2°C/min for separation of trimethylsilyl ether and acetyl derivatives. Analyses of squalene, longer alkylesters, and acylsterols were carried out using a column packed with Diasolid ZT (Nihon Chromato Works Co. Ltd., Tokyo). Column temperatures were 195, 300, and 290°C, respectively.

Each peak revealed in the chart was identified by GC-MS, comparison of the retention times with those of authentic standards, and reference to relative retention times in the literature (Itoh et al 1973).

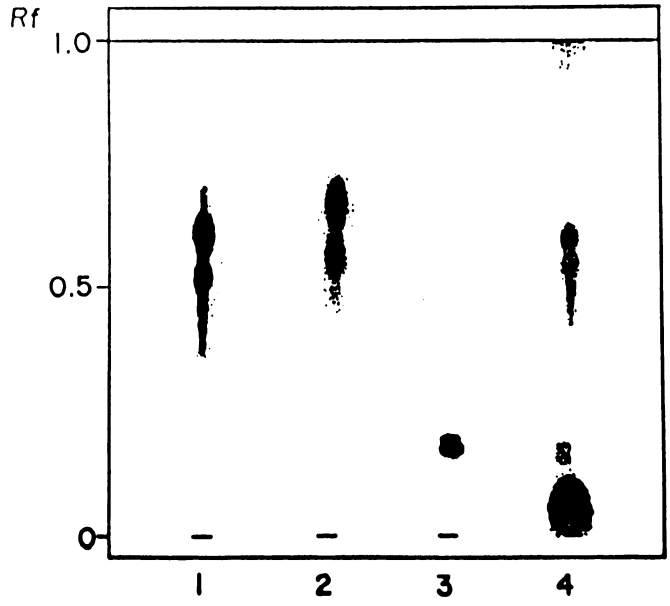

Fig. 1. Thin-layer chromatogram of waxy esters in wheat grain. Developed on silica gel G with hexane/benzene (2:1, v/v) and detected by 50% H_2SO_4. **1,** Acylsterols; **2,** longer alkylesters; **3,** shorter alkylesters; **4,** neutral lipids.

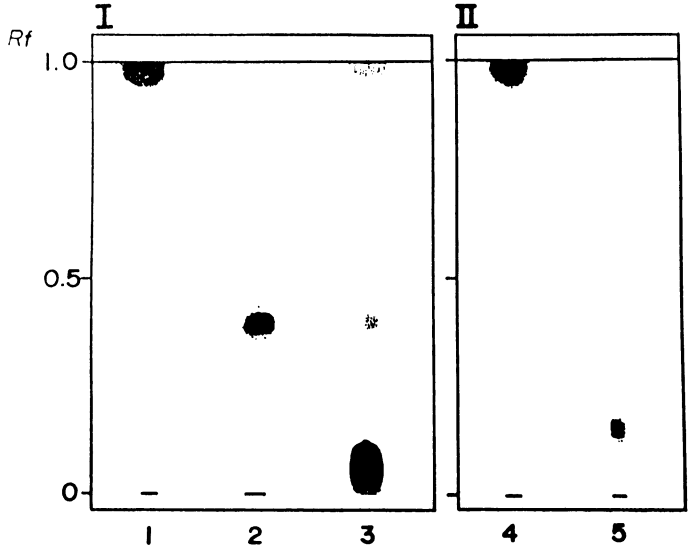

Fig. 2. Thin-layer chromatogram of waxy hydrocarbons in wheat grain. Developed on silica gel G with hexane/benzene (19:1, v/v) for **I** and on silica gel G-$AgNO_3$ (95:5, w/w) with hexane for **II**, and detected by 50% H_2SO_4. **1,** Alkanes and alkenes; **2,** squalene; **3,** neutral lipids; **4,** alkanes; and **5,** alkenes.

GC-MS

GC-MS was performed with a RMU-6MG gas chromatograph-mass spectrometer interfaced with a 002B-8DK computer (Hitachi Seisakusho Co. Ltd., Tokyo). The chromatograph was fitted with a glass column, 0.3 × 100 cm, packed with Diasolid ZT. The operating conditions were the same as described previously (Kuroda et al 1977).

RESULTS

TLC of Waxy Lipids

TLC of waxy esters isolated from wheat grain is shown in Figure 1 and that of waxy hydrocarbons in Figure 2. Contents of acylsterols, shorter alkylesters, hydrocarbons, and longer alkylesters of the neutral lipids were 7.4, 0.5, 0.4, and <0.1%, respectively. The ratio of alkanes, alkenes, and squalene was approximately 79:9:12 in the hydrocarbon fraction.

IR Spectra of Waxy Lipids

IR spectra of acylsterols, longer alkylesters, and shorter alkylesters showed absorptions due to CH_3 (2,960, 2,870, 1,460, and 1,380 cm^{-1}), CH_2 (2,930, 2,850, 1,460, and 720 cm^{-1}), and ester carbonyl groups (1,740 and 1,180 cm^{-1}). That of squalene showed absorptions at 1,665 and 840 cm^{-1} caused by the C=C group in addition to absorptions of CH_3 and CH_2 groups. The IR spectra were in agreement with those reported in the literature (Fathipour et al 1967, Kolattukudy 1970, Kramer et al 1972, Kuroda et al 1977).

Composition of Acylsterols

Major fatty acids of the acylsterols were palmitic and linoleic acids (Table I). It was characteristic that the palmitic acid content of acylsterols was higher than that of triacylglycerols (Ito et al 1984). Sterol composition of acylsterols is shown in Table II. The ratio of 4-desmethylsterol, 4-monomethylsterol, and triterpene alcohol was 85:3:12, 4-desmethylsterol being predominant. The

TABLE I
Fatty Acid Composition (%) of Acylsterols and Shorter Alkylesters in Wheat Grain

Fatty Acid	Acylsterol	Shorter Alkylester	Triacylglycerol[a]
14:0	<0.1	0.2	<0.1
16:0	44.5	22.3	18.1
16:1	2.1	0.6	0.1
18:0	1.3	4.3	1.5
18:1	5.0	13.7	15.0
18:2	42.5	54.2	60.2
18:3	4.6	4.7	5.1
Saturated type	45.8	26.8	19.6
Unsaturated type	52.2	73.2	80.4

[a] From Ito et al 1984.

major 4-desmethysterols were sitosterol and campesterol. Patterns of 4-desmethylsterol in acylsterols, but not of 4-monomethylsterol and triterpene alcohol, were similar to that of free sterol.

The total ion chromatogram from GC-MS analysis of acylsterols exhibited at least four peaks (Fig. 3). Peaks 1 and 3 were also observed by monitoring the sterol fragment ion at a mass-to-charge ratio of m/z 382 because of campesterol, whereas peaks 2 and 4 were detected by monitoring at m/z 396 to account for sitosterol (Ito et al 1983). On the basis of these fragment ions, which were generally the base peaks, together with the data for the component fatty acids and molecular ions detected at m/z 638 for peak 1, m/z 652 for peak 2, m/z 662 for peak 3, and m/z 676 for peak 4, peaks 1–4 were identified as palmitoyl campesterol, linoleoyl campesterol, palmitoyl sitosterol, and linoleoyl sitosterol, respectively, although not all of the acylsterols gave characteristic fragment ions indicating the component fatty acid.

Composition of Shorter Alkylesters

Shorter alkylesters were found to be composed only of methylesters (Ito et al 1981), because the GC peaks did not disappear after methanolysis. The principal fatty acids of the shorter alkylesters were linoleic and palmitic acids. The pattern was similar to that of triacylglycerols (Table I).

Composition of Alkanes and Alkenes

Alkane and alkene compositions in wheat grain are shown in Table III. For both, odd-numbered normal hydrocarbons containing more than 20 carbons were predominant. Major alkanes were nonacosane, heptacosane, and pentacosane; major alkenes were pentacosene, nonacosene, and hentriacontene. Although the position of the double bond in alkenes was not determined, these components were monoene, hence two mass units less than the corresponding alkane. Four minor peaks were also found that were presumed to be branched components of C_{28}, C_{30}, C_{32}, and C_{34} from their retention times on GC. The sums of these peaks were 1.9% of the alkanes and 0.5% of the alkenes.

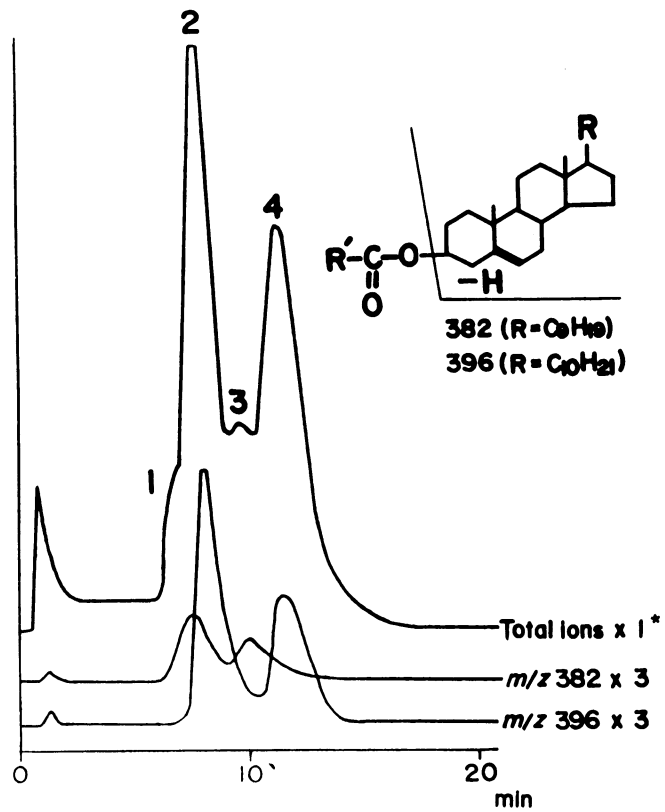

Fig. 3. Chromatograms from gas chromatography-mass spectrometry analysis of acylsterols from wheat grain. A glass column, 0.3 × 100 cm, was packed with Diasolid ZT. Column temperature was 290° C. The energy level of the ion source was 20 eV, and the ionizing current 80 μA. The numerals on the right side (*) show the amplification of the intensity. Peak **1**, R = C_9H_{19} and R′ = palmitoyl; peak **2**, R = C_9H_{19} and R′ = linoleoyl; peak **3**, R = $C_{10}H_{21}$ and R′ = palmitoyl; peak **4**, R = $C_{10}H_{21}$ and R′ = linoleoyl.

Composition of Longer Alkylesters

GC analysis showed that the main component alcohols of the crude longer alkylesters were tetracosanol (39.9%), hexacosanol (20.2%), docosanol (18.7%), and octacosanol (9.1%), whereas component fatty acids were palmitic (19.8%), arachidic (17.1%), behenic (15.0%), and stearic (9.1%).

Nine peaks were identified by GC-MS (Ito et al 1983) as longer alkylesters, from C_{38} to C_{46} (Fig. 4), the main ones being C_{42} (30.8%), C_{44} (27.6%), C_{40} (17.2%), and C_{46} (10.4%). As the major fragment ions, m/z 257 (palmitic acid) and 364 (hexacosanol) were detected for C_{42}, m/z 313 (arachidic acid) and 336 (tetracosanol) for C_{44}, m/z 257 (palmitic acid) and 336 (tetracosanol) for C_{40}, and m/z 341 (behenic acid) and 336 (tetracosanol) for C_{46}. These data indicated that the principal molecular species of longer alkylesters were palmitoyl hexacosanol, arachidoyl tetracosanol, palmitoyl

TABLE II
Sterol Composition (%) of Acylsterols in Wheat Grain

Sterol	Acylsterol	Free Sterol
4-Desmethylsterol		
Cholesterol	<0.1	0.3
Campesterol	22.8	26.0
Stigmasterol	2.0	3.0
Sitosterol	71.8	65.6
Δ^5-Avenasterol	2.9	3.6
Δ^7-Stigmastenol	<0.1	1.0
Δ^7-Avenasterol	0.5	0.5
4-Monomethylsterol		
Lophenol	2.8	0.4
Obtusifoliol	11.0	20.0
Gramisterol	38.5	35.2
Citrostadienol	25.8	18.9
Unknown	21.9	25.5
Triterpene alcohol[a]		
Cycloartanol	···	9.2
β-Amyrin	66.5	32.1
α-Amyrin	32.5	21.0
24-Methylenecycloartanol	···	19.0
Cyclobranol	1.0	8.2
Unknown	···	10.5

[a]Corresponding to 4,4-dimethylsterol.

TABLE III
Composition (%) of Alkanes and Alkenes in Wheat Grain

Carbon Number	Alkane	Alkene
13–19	6.9	19.7
20	0.8	0.3
21	2.3	2.7
22	0.6	1.0
23	1.8	5.6
24	1.0	0.4
25	17.1	16.8
26	3.2	2.4
27	20.7	9.7
28	4.0	5.8
29	22.7	14.7
30	2.4	3.9
31	10.6	12.9
32	1.6	1.1
33	1.4	1.8
34	0.4	0.7
35	0.3	<0.1
36	0.3	<0.1
Others[a]	1.9	0.5

[a]The sum of other minor components, which were all presumed to be branched hydrocarbons (C_{28}, C_{30}, C_{32}, and C_{34}).

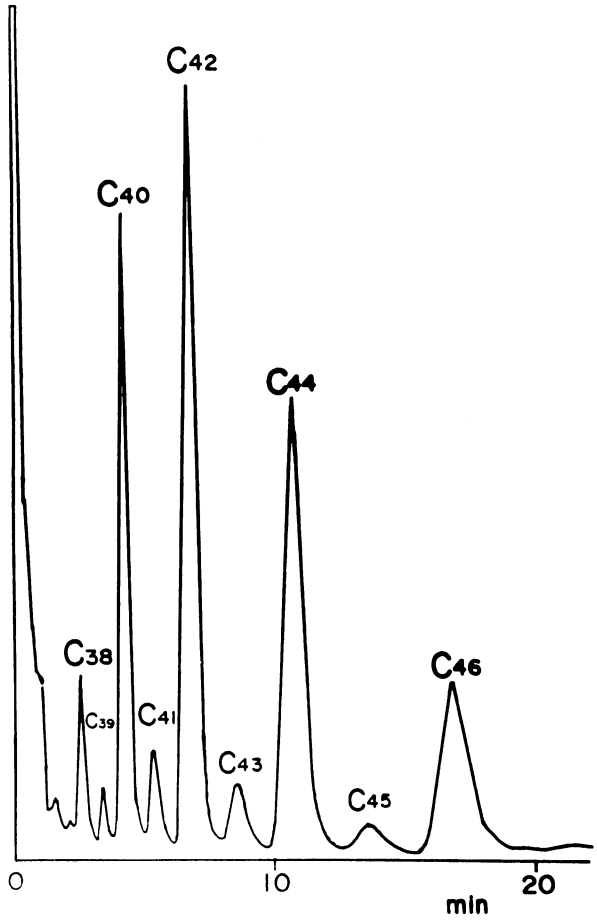

Fig. 4. Gas chromatogram of longer alkylesters from wheat grain.

tetracosanol, and behenoyl tetracosanol. Arachidoyl docosanol, palmitoyl octacosanol, stearoyl docosanol, and arachidoyl hexacosanol were also recognized in the peaks of C_{42}, C_{44}, C_{40}, and C_{46}, respectively.

DISCUSSION

In the literature, hydrocarbons and acylsterols have been reported to be present in waxy lipids of wheat. The presence of shorter and longer alkylesters has been reported for the first time here. In rice bran, longer and shorter alkylesters amounted to 1% of neutral lipids (Ito et al 1983), whereas in wheat grain their contents were <0.1 and 0.5%, respectively. The content of acylsterols was approximately 7% of the neutral lipids, roughly identical to that reported by Morrison (1978). The content of hydrocarbons, 0.4%, was higher than that reported for wheat germ (Kuksis 1964) or wheat flour (Youngs and Gilles 1970, Lorenz and Maga 1975), which may be explained by the fact that we analyzed whole grain.

Fatty acid and sterol compositions of acylsterols were largely identical to those obtained from wheat flour (MacMurray and Morrison 1970). The major alkanes contained 29, 27, and 25 carbons, and principal alkenes contained 25, 29, and 31 carbons. This may be the general pattern in higher plants (Kolattukudy 1980), although a C_{31} alkane and a C_{33} alkene were predominant in rice bran (Ito et al 1981). The presence of cyclohexyl hydrocarbons (Kuksis 1964, Youngs and Gilles 1970) could not be confirmed in the present study. The longer alkylesters we found were in the range of C_{38} to C_{46}; these are shorter than those in plants in general (Kolattukudy 1980) or in rice bran (Ito et al 1983). Shorter alkylesters in wheat grain were composed only of methylesters and not of ethylesters, as found in rice (Ito et al 1983). The fatty acid composition of shorter alkylesters was also different from that of rice bran.

These results suggested waxy lipids in one wheat variety grown in Japan differed considerably from those in rice, even though the two cereals belong to the same family.

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