Aroma Constituents of Soybean [*Glycine max* (L.) Merril] Milk Lacking Lipoxygenase Isozymes

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The soybean milk odor of samples lacking lipoxygenases (LOs) 2 and 3 and LOs 1, 2, and 3, was concentrated by simultaneous distillation and extraction (SDE), together with that of normal soybean milk. The constituents of the odorants were identified by GC and GC–MS data. The yields of volatile compounds were greatly decreased when the lipoxygenases were lacking, and the changes of each constituent were discussed. From the aroma extract dilution analysis, the C_8, C_9, and C_{10} alcohols and carbonyl compounds were thought to be important to address the odor of LO-deficient soybean milk. The amount of 1-octen-3-ol did not vary among the soybean samples and the 3-*R*-(−)-configuration was established, which suggest that this compound is also formed by enzymatic reaction.

Keywords: *Soybean; lipoxygenase; aroma concentrate; chirality; aromagram*

INTRODUCTION

Soybeans have long been used in Asian countries as a food in the traditional forms of tofu, miso, shoyu, natto, and tempeh. Soybeans have recently become one of the most economical sources of food protein, and soybean milk, sherbet, and ice cream have been produced as substitutes for the dairy products. However, soybean milk products have a beany and grassy off-flavor that has inhibited the wider use of this economical protein source.

The formation of such an off-flavor has been widely investigated, and it has been established that C_6 alkyl- or alkenylaldehydes are the main constituents of the odor, which are formed by oxidation and breakdown of unsaturated fatty acid molecules (Rackis *et al.*, 1979).

Soybeans are rich in lipoxygenase (LO) content consisting of the three isozymes, 1, 2, and 3, which are active under different conditions and different states of the substrate to yield hydroperoxides, and subsequent cleavage by hydroperoxide lyase gives C_6 aldehydes (Axelrod *et al.*, 1981; Siedow, 1991; Zhuang *et al.*, 1991).

The formation mechanism for C_6 aldehydes in plants and the reduction of C_6 aldehydes to the corresponding alcohols have also been reviewed recently (Hatanaka, 1993). The precursors in soybean seed to produce the off-flavor substance by enzymatic oxidation and cleavage are polyunsaturated fatty acids, particularly $C_{18:2}$ linoleate to produce hexanal and $C_{18:3}$ linolenate to produce (*E*)-2-hexenal and (*Z*)-3-hexenol via (*Z*)-3-hexenal.

These fatty acids are present as triglycerides, as esters of phospholipids, and as free acids; therefore, it is difficult to inhibit the formation of the off-flavor by excluding the flavor precursors from soybean seeds. Soybeans lacking various lipoxygenase isozymes were recently found (Hildebrand and Hymowitz, 1981) and were shown to produce limited amounts of C_6 aldehydes and alcohols. Since that time, breeding for new isolines of a soybean cultivar lacking LO-1, LO-2, and/or LO-3 has progressed (Pfeiffer *et al.*, 1992). In Japan, one of the lines lacking both LO-2 and LO-3 has been registered as a new soybean cultivar named Yumeyutaka, which has shown a much lower off-flavor and better taste than the parent Suzuyutaka, a leading cultivar in Japan (Kitamura *et al.*, 1992; Kitamura, 1993). More recently, a mutant soybean lacking LOs -1, 2, and 3 has been cultivated, and it exhibited normal growth and seed production (Takamura *et al.*, 1991); the new cultivar has been named Kyushu No. 111.

Using normal and LO-deficient lines of soybeans, the activities of specific lipoxygenase isozymes have been evaluated (Zhuang *et al.*, 1991). In this paper, the aroma constituents of three lines of soybean, normal cultivar Suzuyutaka, LO-2- and LO-3-deficient Yumeyukata, and the new cultivar Kyushu No. 111 lacking LOs -1, 2, and 3, are analyzed chemically by gas chromatography, and each gas chromatogram is analyzed to correlate with the flavor character. The chirality of 1-octen-3-ol is also discussed, which is an important constituent among the aroma concentrates obtained from the three different soybeans.

EXPERIMENTAL PROCEDURES

Materials. Suzuyutaka containing lipoxygenases 1, 2, and 3 (abbreviated +LO-1,2,3 hereafter), Yumeyutaka lacking lipoxygenases 2 and 3 (abbreviated −LO-2,3), and Kyushu No. 111 lacking lipoxygenases 1, 2, and 3 (abbreviated −LO-1,2,3) were harvested in 1993 and preserved under the normal conditions at 5–7 °C.

Separation of the Volatile Compounds from Soybean Seeds. Soybean seeds (55 g) were soaked overnight in 300 mL of water and then homogenized in a mixer for 3 min. After 0.1 mL of an ethereal solution of methyl decanoate (0.6 mg/mL of ether) was added as an internal standard, the volatiles were extracted by the simultaneous distillation and extraction method (SDE), using a modified Likens–Nickerson apparatus with 50 mL of ether as the extracting solvent. After 30 min of extraction, the ethereal solution was dried over Na_2SO_4 and condensed to *ca.* 10 μL by distillation and blowing with N_2 gas. This extract was treated as the aroma concentrate.

Gas Chromatography (GC) and GC–Mass Spectrometry (GC–MS). A Hewlett-Packard 5890 gas chramatograph

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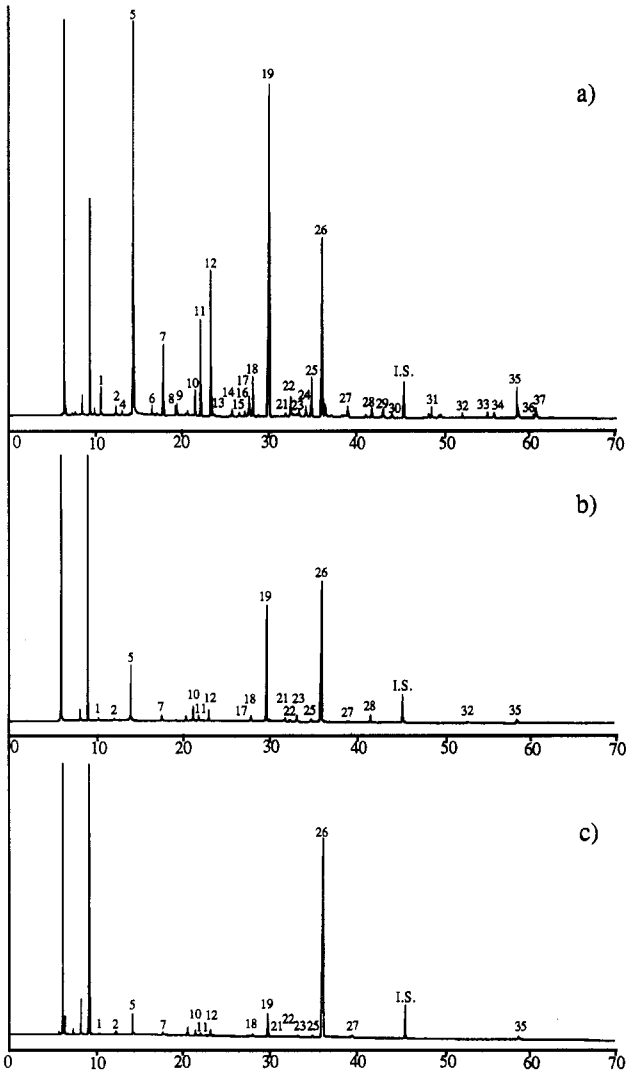

Figure 1. Gas chromatograms of the aroma concentrate obtained from three different types of soybean: (a) Suzuyutaka (+LO-1,2,3); (b) Yumeyutaka (−LO-2,3); (c) Kyushu No. 111 (−LO-1,2,3); I.S., methyl decanoate.

equipped with a flame ionization detector was used. The GC conditions were as follows: WCOT column of DB-Wax, 60 m × 0.25 mm (i.d.); carrier gas (He) flow rate, 1.2 mL/min; oven temperature, programmed from 60 (4-min hold) to 180 °C at 2 °C/min; injection and detection temperature, 200 °C. The peak area percentages and Kovats indices (KI) were calculated by a Shimadzu Chromatopac C-R6A integrator. The GC conditions for the GC−MS analysis were the same as those described for GC, the mass spectrometer being used in the EI mode with an ionization voltage of 70eV. The MS data were processed by a JEOL-DA 5000 system, and each compound was identified by comparing MS data and KI values to those of standards and literature data.

GC Separation of the Chiral Compound. The optical resolution of (*R*)- and (*S*)-1-octen-3-ol was assessed by using a WCOT column coated with permethylated α-cyclodextrin (α-DEX 120 column, Supelco). The racemate of commercially available 1-octen-3-ol was separated into two peaks with the same intensity. The authentic (*R*)- and (*S*)-1-octen-3-ols were offered from the Technical Research Institute of Hasegawa Perfumery Co., Japan. A direct comparison of the KI value of natural 1-octen-3-ol and cochromatography with authentic optical isomers were applied to establish the absolute configuration of the natural product.

Gas Chromatography Olfactometry (GCO). An aroma extract dilution analysis (AEDA; Grosch, 1993) was applied to create an aromagram of each soybean aroma concentrate, which was diluted stepwise with diethyl ether in a volume ratio of 1:10^n, where n was selected from 1, 2, or 3 as the flavor dilution (FD) factor. Aliquots (0.5 μL) of a diluted sample were analyzed by GC under the conditions already described, and the GC effluents were evaluated by using an odor sniffing system. Odor threshold values were determined for the respective FD factors at which the odor of the effluent could still be smelt.

Table 1. Identified Compounds in the Aroma Concentrates Obtained from Three Different Types of Soybean Milk

peak no.	identified compound	Kovats index found	Kovats index std	relative peak area[a] +LO-1,2,3	relative peak area[a] −LO-2,3	relative peak area[a] −LO-1,2,3
1	pentanal	967	967	42.1	3.4**	0.0*
2	chloroform[b]	1025				
4	propanol	1042		11.5	0.0*	0.0*
5	hexanal	1076	1071	1513.1	114.3*	40.9*
6	(*E*)-2-pentenal	1121		28.3	6.7***	2.5**
7	1-penten-3-ol	1147	1145	137.1	4.7*	0.0*
8	2-heptanone	1172	1168	18.5	0.0*	0.0*
9	heptanal	1175	1171	25.7	0.0*	0.0*
10	(*E*)-2-hexenal	1209	1206	62.3	42.7	13.3*
11	2-pentylfuran	1221	1213	240.0	15.7*	0.0*
12	1-pentanol	1242	1243	381.8	22.3*	2.6*
13	3-octane	1244	1243	7.5	0.0*	11.5*
14	ni[c]	1278		20.4	0.0*	0.0*
15	1-octen-3-one	1290		14.2	0.0*	0.0*
16	(*E*)-2-pentenol	1299		15.3	0.0*	0.0*
17	(*Z*)-2-pentenol	1308		85.6	3.9*	0.0*
18	(*E*)-2-heptenal	1314		94.2	14.1**	3.7*
19	hexanol	1348	1341	1590.7	406.7*	59.3*
21	(*Z*)-3-hexenol	1374	1378	11.8	11.0	0.0***
22	nonanal	1382	1387	85.6	9.3*	4.7*
23	(*E*)-3-octen-2-one	1396		21.8	20.2	0.0**
24	ni	1408		32.2	0.0*	0.0*
25	ni	1419		111.2	9.4*	0.0*
26	1-octen-3-ol	1440	1440	629.0	447.5	732.8
27	(*E*,*E*)-2,4-heptadienal	1482	1479	30.0	0.0	4.2
28	(*E*)-2-nonenal	1525	1521	31.0	33.9**	0.0*
29	octanol	1546	1551	26.0	0.0*	0.0*
30	ni	1561		11.2	0.0*	0.0*
31	(*E*)-2-decenal	1633	1637	41.8	0.0**	0.0**
32	(*E*,*E*)-2,4-nonadienal	1691	1693	20.0	0.0**	0.0**
33	(*E*)-2-undecenal	1743	1741	25.0	0.0***	0.0***
34	(*E*,*Z*)-2,4-decadienal	1757		30.9	0.0***	0.0***
35	(*E*,*E*)-2,4-decadienal	1805	1801	159.3	14.2*	9.4*
36	hexanoic acid	1836	1844	10.1	0.0**	0.0**
37	4-ethoxybenzaldehyde	1844		42.3	0.0	0.0
	total			5607.5	1180.0	884.9
	ratio			6.3 :	1.3 :	1.0

[a] Significant differences compared with normal soybean value at P = <0.001 (*), <0.01 (**), and <0.05 (***), respectively (Student's t-test). [b] Thought to be a contaminant from solvent. [c] ni, not identified with a known compound.

RESULTS AND DISCUSSION

The gas chromatograms of the aroma concentrates obtained from soybeans +LO-1,2,3 (normal), −LO-2,3, and −LO-1,2,3 are shown Figure 1, where the peak numbers correspond to the identified compounds that are summarized in Table 1. The relative peak area to that of the internal standard (I.S. peak area = 100) is the average from three runs for separation and GC analysis with each soybean sample. The significant difference of a specific compound among the three different samples was calibrated by Student's t-test.

From Figure 1 and Table 1, the main constituents in the volatiles of normal soybean milk were hexanal, 1-penten-3-ol, (*E*)-2-hexenal, 2-pentylfuran, 1-pentanol, (*Z*)-2-pentenol, (*E*)-2-heptenal, hexanol, nonanal, 1-octen-

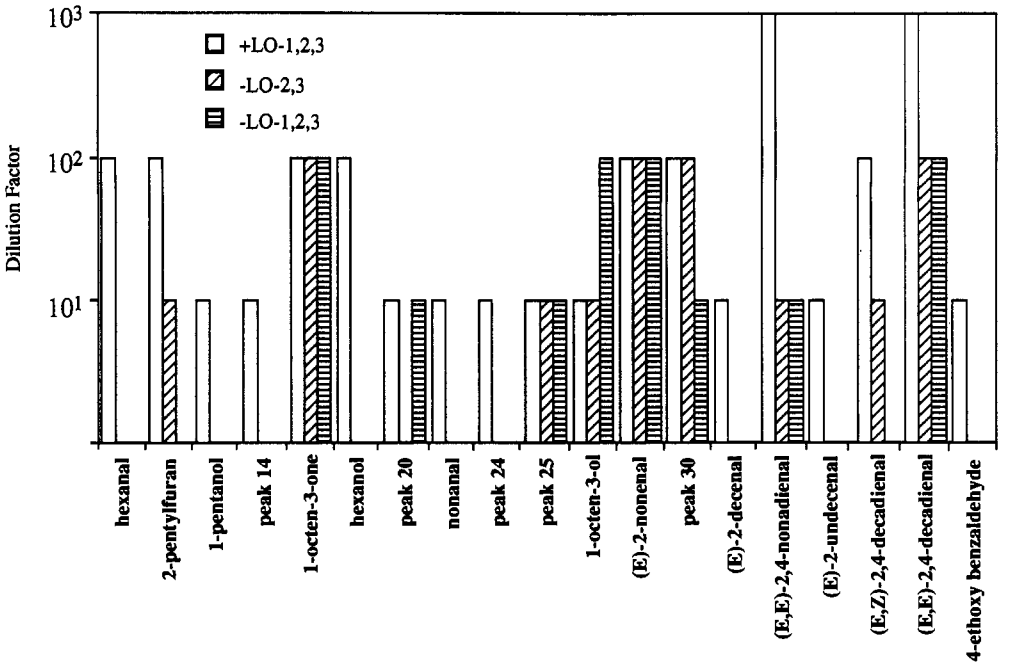

Figure 2. Histogram of the FD factors for 19 representative odorants in soybean milk.

3-ol, and (*E,E*)-2,4-decadienal, all of which are thought to be degradative oxidation products of polyunsaturated acids.

The whole peak areas of −LO-2,3 and −LO-1,2,3 were much less than that of normal +LO-1,2,3 soybean. In −LO-1,2,3, all of the main peaks, except for 1-octen-3-ol were significantly less, resulting in one-sixth of the total peak area of normal soybean. On the other hand, the large peaks of (*E*)-2-hexenal, (*Z*)-3-hexenol, (*E*)-3-octen-2-one, 1-octen-3-ol; and (*E*)-2-nonenal were not much less in −LO-2,3, although the total peak area was one-fifth that of standard soybean. This result suggests that isozyme LO-1 oxidized linolenate to yield an unsaturated C_6 alcohol and aldehyde, although the quantitative decrease in the volatiles of soybean milk is attributable to the deficiency of LO-2 and LO-3. Zhuang *et al.* found that LO-2 and LO-3 oxidize unsaturated acid in the form of ester and LO-1 oxidizes free unsaturated fatty acid; therefore, the main odor components of soybeans, hexanol and hexanal, are thought to be oxidation products of linoleic acid which are present in the form of esters and are oxidized by LO-2 and LO-3.

A sensory evaluation of soybean products made from normal and lipoxygenase-deficient soybean has already been made by many researchers (Matoba *et al.*, 1985; Davies *et al.*, 1987); however, the contribution of a particular compound in the volatiles to the whole grassy−beany odor of soybean milk had not been established. We applied an AEDA (Grosch, 1993) to the soybean aroma concentrates. As shown in Figure 1, the three GCs each had approximately the same peak height for the internal standards, meaning that the GC for each aroma concentrate represents its quantitative aroma composition. They were diluted with 10, 10^2, and 10^3 times the volume of ether, and after each dilution step, the same amount of solution was injected into the gas chromatograph to perform the GCO.

The aromagrams from the three soybean samples (+LO-1,2,3 as the control, −LO-2,3 and −LO-1,2,3) by GCO are summarized in Figure 2 as a bar chart of three FD factors for 19 representative odorants of soybean milk. This histogram shows that hexanal and hexanol, which contribute to the greenish odor of normal soybean, had disappeared completely at the first dilution stage, which supports the earlier assertion that the lower level of hexanal is the main reason of the odor improvement of LO-deficient soybean (Matoba *et al.*, 1985). (*E,E*)-2,4-Nonadienal and (*E,Z*)-2,4-decadienals showed strong odor in +LO-1,2,3 and moderate odor in −LO-2,3 in spite of the fact that these compounds did not appear on the gas chromatogram of the latter. The inconsistency between those aromagrams and gas chromatograms can be explained from the low threshold values of these compounds. On the other hand, (*E,E*)-2,4-decadienals were recognized on both aromagrams and gas chromatograms of all samples. These results suggest that C_9 and C_{10} dienals are thought to be important for the LO-lacking soybean flavor. 1-Octen-3-one, 1-octen-3-ol, (*E*)-2-nonenal, and unidentified peak 30 have high FD factors and, except for 1-octen-3-ol, the peaks of these compounds were very small or could not be recognized on the relevant gas chromatogram. For example, peak 20 is not listed in Table 1, but it showed characteristic aroma character and FD value compared to those of neighboring peaks. Therefore, these compounds would have very low threshold values and exert a secondary effect on soybean odor. C_8 alcohol and ketone, as well as C_9 and C_{10} compounds, are thought to be important to the odor of soybean products made from LO-deficient lines.

1-Octen-3-ol is interesting because it shows the same yield from the three types of soybean. This compound is known to be a key ingredient of mushroom aroma, and the absolute configuration of the natural compound has been established as *R*-(−) (Würzenherger and Grosch, 1984). As shown in Figure 3a, the single peak of 1-octen-3-ol appeared on the GC trace of the natural soybean aroma when using an optically active α-DEX column, this being identical with the latter peak of the standard racemate (Figure 3b). The elution sequence of *S* before *R* on this GC was traced by comparison with authentic (*R*)- and (*S*)-1-octen-3-ols, and the absolute configuration of soybean 1-octen-3-ol as *R* was established by cochromatography of the natural one with the standard racemate (Figure 3c).

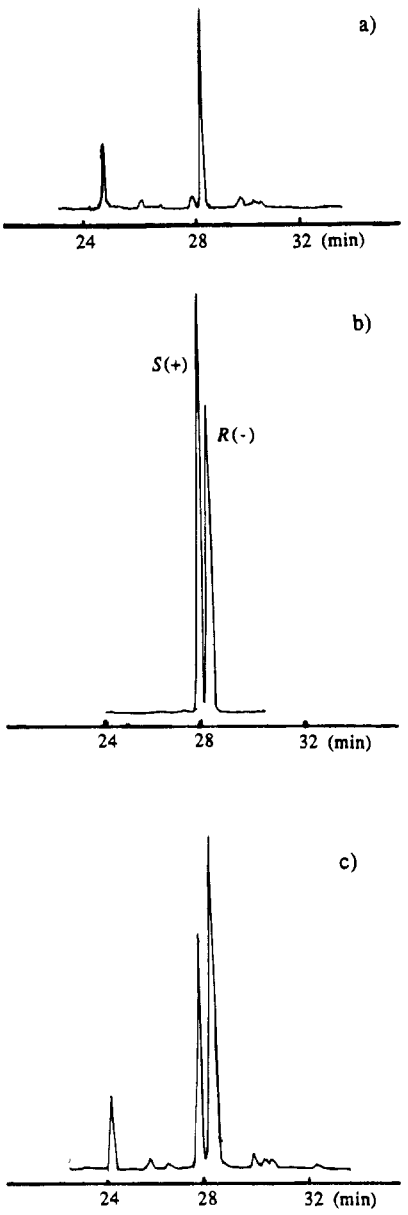

Figure 3. Optical resolution of 1-octen-3-ol by gas chromatography: (a) natural flavor component; (b) synthetic racemate; (c) natural + synthetic racemate.

The presence of as much (*R*)-1-octen-3-ol in −LO-1,2,3 soybean milk as in the standard sample suggests that 10-hydroperoxide is formed by other hydroperoxidation than that by LO-1, LO-2, and LO-3, because the hydroperoxide lyase is thought to be present in all types of these soybean cultivers.

Moreover, the chiral configuration of 1-octen-3-ol from soybean is the same as that found from mushroom (Würzenberger and Grosch, 1984) and the (*R*)-configuration of 1-octen-3-ol has a stronger mushroom-like odor (Mosandl, 1986). A chemical study to identify the two unknown compounds (peaks 20 and 30), which were flavor-contributing compounds screened by AEDA, is now in progress.

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