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Differences in the Amount and Range of Volatile Carbonyl Compounds Formed by Lipoxygenase Isoenzymes from Soybeans

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The lipoxygenases from soybeans, L-1 (optimum pH 9.0), L-2 (pH 6.5), and L-3 (pH 6.5), were incubated with linoleic and with linolenic acid. The carbonyl compounds formed were isolated and analyzed as 2,4-dinitrophenylhydrazones. The experiments showed that L-2 and L-3 form significantly more carbonyl compounds than the alkaline enzyme L-1. From reactions with linolenic acid as substrate the relative amounts of carbonyl compounds which were formed during the catalysis were estimated (mole percent). L-3 formed propanal (41), 2-*trans*-pentenal (11), 2-*trans*-hexenal (9), 2-*trans*,6-*cis*-nonadienal (2.5), 2-*trans*,4-*cis*-heptadienal (20), 3,5-octadien-2-one (8), and 2,4,6-nonatrienal (8.5). In the experiment with the other neutral lipoxygenase a similar range of carbonyl compounds was identified. In contrast, only 2-*trans*-hexenal (77), propanal (18), and 2-*trans*-pentenal (5) arise during the incubation of linolenic acid with L-1.

Various volatile carbonyl compounds which arise from enzymatic oxidative breakdown of unsaturated fatty acids are detected in the aroma of fruits and vegetables (Drawert et al., 1966, 1973; Grosch and Schwarz, 1971). These volatile aldehydes and ketones only occur in plants in extremely small concentrations, more of them being formed if the cell has broken so that oxygen can penetrate the tissue.

To get an insight into the formation of volatile carbonyl compounds we have begun to study model systems containing an unsaturated fatty acid and a factor which could possibly promote lipid peroxidation in plant tissues. In a first paper (Grosch et al., 1974) we have compared the patterns of volatile carbonyl compounds arising from linolenic acid by autoxidation (accelerated by haemoglobin), by singlet oxygen and in the presence of lipoxygenase. In these experiments we have used a purified lipoxygenase isoenzyme from peas (pH optimum 6.5). However, not only neutral lipoxygenases occur in plants. For example, soy beans and peanuts contain additionally an alkaline isoenzyme (pH optimum 8.5–9.0) (Christopher et al., 1970, 1972a; Dillard et al., 1960).

Studies on the lipoxygenase isoenzymes of soybeans have shown that they differ not only in pH optimum but also in their power to co-oxidize carotenoids (Weber et al., 1974) and in substrate (Christopher et al., 1970, 1972a) and peroxidation specificities (Christopher et al., 1972b; Leu, 1974; Roza and Francke, 1973). In view of the formation of volatile carbonyl compounds it is of interest to investigate the neutral and alkaline lipoxygenase isoenzymes from soybeans in relation to their capacity for promoting the breakdown of unsaturated fatty acids to such flavor compounds. This article deals with the observed qualitative and quantitative differences.

EXPERIMENTAL SECTION

Materials and Reagents. The following materials were used: soybeans (Harburger Ölwerke, Brinckmann and Mergell); linolenic acid (>99%, Nu Chek Prep); Tween 20 and 80 (Schuchardt); piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes buffer, Sigma); Al_2O_3 neutral (Woelm); Seasorb 43 (Fisher Scientific); 2,4-dinitrophenylhydrazine (DNPH; Merck), recrystallized from benzene; *n*-heptane (Merck) was freed from carbonyl compounds as described for *n*-hexane (Grosch, 1968). The other chemicals were analytical grade.

Separation of Lipoxygenases-1, -2, and -3 from a Soy Extract. The lipoxygenase isoenzymes were separated by DEAE-cellulose chromatography as previously described (Weber et al., 1974). For clear designation of the isoenzyme an elution diagram is shown in Figure 1. The fractions containing an isoenzyme were collected and the protein content calculated from the extinction at 280 nm with $E_{1\ cm}^{1\%}$ of 14.2.

Determination of the Lipoxygenase Activity. After DEAE chromatography the collected isoenzymes were assayed at 23° at pH 6.5 (L-2 and L-3) and at pH 8.5 (L-1) using a modification of the Surrey substrate (Surrey, 1964). The assay mixture contained 1.65 m*M* linoleic acid, 0.5 μl/ml of Tween 20, 0.1 *M* sodium phosphate buffer (pH 6.5), or 0.1 *M* sodium borate buffer (pH 8.5). In 3 ml of reaction mixture one unit caused a $\Delta E_{234}^{1\ cm}$ of 1.0 between 30 and 60 sec.

Oxidation Experiments. *Fatty Acid Emulsion.* One-hundred milligrams of linoleic acid or linolenic acid was (with the addition of 5 ml of 0.001% Tween 80 and some drops of 1 *N* NaOH) dissolved in 20 ml of H_2O. The solution was diluted to 360 ml with 0.025 *M* Pipes buffer (pH 6.5) or 0.025 *M* Tris-HCl buffer (pH 8.5). The pH of the emulsion was corrected to 6.5 or to 8.5 with dilute HCl.

Incubation. Fatty acid emulsion (360 ml), pH 6.5 or 8.5, was cooled in a 1000-ml round-bottomed flask to 10° and degassed with O_2 (5 min). L-3, L-2, or L-1 (dissolved in 40 ml of 0.01 *M* sodium phosphate buffer (pH 7.0)) was added to the emulsion and the flask closed. The reaction mixture was stirred for 20 min at 10°. (The incubation was carried out at a low temperature to hinder a breakdown of hydro-

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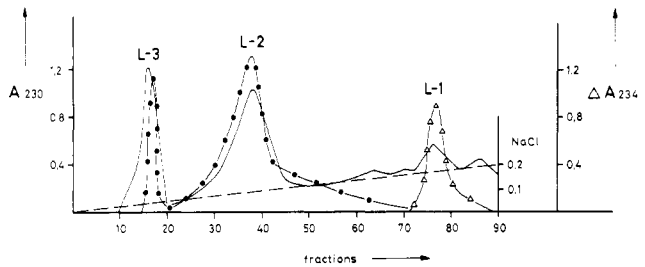

Figure 1. DEAE-cellulose chromatography of an extract from soybeans (Weber et al., 1974): (●) lipoxygenase activity at pH 6.5; (△) lipoxygenase activity at pH 9.0; (—) protein.

peroxides.) Immediately after incubation the pH was changed to pH 8 with 1 *N* NaOH; this step was omitted in the incubation experiment with L-1. This was followed by the addition of 10 ml of methanol and 10 ml of $CaCl_2$ solution (3 g of $CaCl_2 \cdot 2H_2O$ dissolved in 10 ml of H_2O). The precipitate formed was filtered off (refrigerator) and the filtrate acidified with 5 ml of cooled 85% H_3PO_4. DNPH (50 mg) dissolved in 15 ml of cooled 85% H_3PO_4 was added. Before and after the incubation 0.5 ml of the reaction system was pipetted into 3 ml of methanol. After dilution to 5 ml with water the extinction at 234 nm was measured. The diene formation was determined by using a molar extinction coefficient at 234 nm of 25,000 l. mol^{-1} (Johnston et al., 1961). The isolation and identification of the 2,4-dinitrophenylhydrazones (DNP) were carried out as described in an earlier paper (Grosch et al., 1974).

RESULTS

Influence of the Detergent Concentration on the Isolation of DNP. Before carrying out the incubation experiments the procedure for the isolation of the carbonyl compounds was studied. The reaction system containing linolenic acid was the same as described in the Experimental Section, the only difference being the omission of the lipoxygenase isoenzyme and the addition of 10 μmol of hexanal. The analysis of the carbonyl compounds showed that at a concentration of Tween as high as in the photometric assay for lipoxygenase activity (0.5 μl/ml) the hexanal–DNP stayed in solution. Only at a very low detergent concentration (1.25×10^{-2} μl/ml) was a precipitate of hexanal–DNP formed, which could be isolated through filtration with a glass frit. In this experiment 80% of the hexanal was recovered and thus in the following oxidation experiments 1.25×10^{-2} μl/ml of Tween 80 was used.

Amounts of Monocarbonyl Compounds Formed. In the oxidation experiments 340 units of each isoenzyme were incubated with the unsaturated fatty acids. Although the specific activities found in the photometric assay were equal, there was a large difference between the diene formations (Table I). It is primarily the fatty acid hydroperoxides which are clarified by measurement of the diene absorbance, since they arise as main products of the catalysis (Hamberg and Samuelsson, 1967). However, other compounds without diene structure are also formed (Arens and Grosch, 1974; Evans et al., 1967). Although the exact proportion of nondiene products which arise during the catalysis of the three lipoxygenases is unknown, one can infer from the oxidation experiments of Table I that L-1 reacted with more linoleic and linolenic acid than did the neutral lipoxygenases L-2 and L-3.

As described above the Tween concentration in the oxidation experiments is much lower than in the photometric test which was used for the determination of the lipoxygenase activities. The results of Table I give rise to the question whether the small concentrations of Tween aid the specific activity of the L-1 preparations more than the specific activities of L-2 and L-3. A measurement of the initial velocity of the lipoxygenase catalysis under the different conditions of the activity assay (experiment 1 in Table II) and the oxidation experiments (experiments 2 and 3 in Table II) was therefore undertaken. The initial velocity of the lipoxygenase catalysis is a function of the hydroperoxide concentration at the beginning of the reaction (Smith and Lands, 1972). Therefore in this study the enzymes were tested with the same freshly prepared substrate emulsions.

Table II shows that lowering the Tween concentrations does not lead to a substantial reduction in the specific activities of L-2 and L-3 in comparison to the specific activity of L-1. On the contrary the specific activity of the L-3 preparation gains more through the lowering of the Tween concentrations than does the specific activity of the L-1 preparation.

L-1 is more stable than the neutral lipoxygenases (Christopher et al., 1970). We assume that L-2 and L-3 denature more and more during the 20-min incubation procedure and therefore oxidize less linoleic and linolenic acid than L-1. Although L-2 and L-3 form a smaller quantity of dienes than L-1 nevertheless in the presence of the neutral lipoxygenases more carbonyl compounds arise than in the presence of the alkaline lipoxygenase (Table I).

This difference is particularly obvious in the experiment with linolenic acid as substrate. L-1 forms only 1.5 μmol while L-2 and L-3 each form about 7 μmol of carbonyl compounds. The differences between L-2–L-3 and L-1 are not dependent upon the type of buffer ion. Experiments in which a phosphate buffer took the place of Pipes and a borate buffer took the place of Tris also gave the results shown in Table I.

Monocarbonyl Compounds from Linolenic Acid. From three experiments with L-1, 4.5 μmol of monocarbon-

Table I. Amounts of Monocarbonyl Compounds Formed by L-1, L-2, and L-3

	Lipoxygenase isoenzyme in the reaction system				
	Units	mg of protein	Substrate[a]	Substrate converted to dienes,[b] %	Monocarbonyl compd, μmol
L-1 (pH 8.5)	340	4.2	18:2	81	1.5
L-2 (pH 6.5)	340	3.3	18:2	42	3.6
L-3 (pH 6.5)	340	2.6	18:2	56	3.3
L-1 (pH 8.5)	340	5.3	18:3	85	1.5
L-2 (pH 6.5)	340	2.0	18:3	32	6.9
L-3 (pH 6.5)	340	2.2	18:3	30	7.1

[a] 18:2, linoleic acid; 18:3, linolenic acid. [b] From diene absorption.

Table II. Initial Velocity[a] of L-1, L-2, and L-3 with Linoleic and Linolenic Acid as Substrates (Temperature 10°)

Substrate	Specific activity, %		
	L-1 (pH 8.5)	L-2 (pH 6.5)	L-3 (pH 6.5)
(1) 14.6 m*M* linoleic acid + 0.5 μl/ml of Tween 20	100	100	100
(2) 0.9 m*M* linoleic acid + 1.25×10^{-2} μl/ml of Tween 80	100	88	153
(3) 0.9 m*M* linolenic acid + 1.25×10^{-2} μl/ml of Tween 80	147	196	370

[a] The reaction system of 10 ml contained the following amounts of protein: 90 μg (L-1), 106 μg (L-2), and 157 μg (L-3). The velocity between 45 and 90 sec of catalysis was determined polarographically.

Table III. Results of the Analysis of the Monocarbonyl Compounds (as DNP) from Linolenic Acid

Compd no.	Mobility in relation to alcanal–DNP,[a] carbon no.	λ_{max} in $CHCl_3$, nm	Position in $AgNO_3$–TLC	Mol wt[b]	Results of identification
			Oxidation with L-2 or L-3		
1a	2	355			Acetaldehyde[c]
2a	3	358		238	Propanal
3a	3–4	375	2-*trans*-Pentenal	264	2-*trans*-Pentenal
4a	4–5	375	2-*trans*-Hexenal	278	2-*trans*-Hexenal
5a	6	375	2-*trans*,6-*cis*-Nonadienal		2-*trans*,6-*cis*-Nonadienal[d]
6a	3–4	390	2-*trans*,4-*cis*-Heptadienal	290	2-*trans*,4-*cis*-Heptadienal
7a	7–8	393	Two spots	304	3,5-Octadien-2-one
8a	3–4	410	Two spots	316	2,4,6-Nonatrienal
			Oxidation with L-1		
1b	3	358			Propanal
2b	3–4	375	2-*trans*-Pentenal		2-*trans*-Pentenal
3b	4–5	375	2-*trans*-Hexenal	278	2-*trans*-Hexenal

[a] Mobility on thin-layer chromatography (TLC); Kieselgur–Carbowax–cyclohexane. [b] Determined by mass spectrometry of the DNP. [c] Only detected in the experiment with L-2. [d] Only detected in the experiment with L-3.

yl compounds was isolated as DNP; 3.6 μmol gave three compounds which could be identified as described in Table III. A greater number of carbonyl compounds are formed by the neutral lipoxygenases. About 7 μmol was taken for analysis of which 6 μmol (experiment with L-3) and 5.7 μmol (experiment with L-2) were identified as described in Table III. L-2 and L-3 form mainly the same carbonyl compounds. The mass spectrum of compound **7a** agrees with a mass spectrum of 3,5-octadien-2-one DNP (Badings, 1970). Compound **8a** was eluted from the Seasorb–Celite column after the 2,4-alcadienal fraction. The uv spectrum for this compound indicates three double bonds in conjunction with the carbonyl function. The mass spectrum (Figure 2) shows a moleculer peak at *m/e* 316. Compound **8a** was therefore identified as 2,4,6-nonatrienal. On silica gel–$AgNO_3$ the **7a** and **8a** DNP compounds were separated into two spots lying very close to each other. The mass spectra of the two **7a** DNP compounds were very similar to each other; this was also found for the two **8a** DNP compounds. It could therefore be concluded that both **7a** and **8a** are made up of two stereoisomers.

The results of the quantitative analysis are shown in Table IV. According to these L-1 forms primarily 2-*trans*-hexenal whereas for L-2 and L-3 propanal predominates with noticeable quantities of 2-*trans*,4-*cis*-heptadienal, 2-*trans*-hexenal, and 2-*trans*-pentenal. In this respect the neutral soy lipoxygenases are very similar to the lipoxygenase from peas (Grosch et al., 1974). The only difference occurs with 2-*trans*-hexenal which arises in much smaller quantities from the pea lipoxygenase than from the L-2 and L-3.

DISCUSSION

The results show that the neutral lipoxygenases L-2 and L-3 form both a greater quantity and a greater range of volatile carbonyl compounds than does the alkaline isoenzyme L-1. This difference between L-1 and L-2–L-3 correlates with their peroxidation specificities and co-oxidation potentials. At optimum pH L-1 oxidizes linoleic acid only to the 13-hydroperoxide, whereas the neutral lipoxygenases form the 9- and 13-hydroperoxides in an approximate relationship of 1:1 (Christopher et al., 1972b; Roza and Francke, 1973). The neutral enzymes produce a greater range of by-products (unpublished results) and can co-oxidize carotenoids. In contrast, the alkaline isoenzyme is a poor carotenoid "oxidase" (Weber et al., 1974).

In the first step of lipoxygenase catalysis peroxyl radicals, which are mainly converted to hydroperoxides, are formed (De Groot et al., 1973). However, a small amount of singlet oxygen also arises (Finazzi-Agro et al., 1974). Badings (1970) has assumed that during the autoxidation of an unsaturated fatty acid the formation of volatile carbonyl compounds takes place through a breakdown of hydroperoxides. This would mean that the experiment with L-1 should have resulted in the greatest amount of carbonyl compounds because L-1 oxidized considerably more linoleic and linolenic acid to hydroperoxides than L-2 and L-3. However, our results do not endorse this hypothesis since

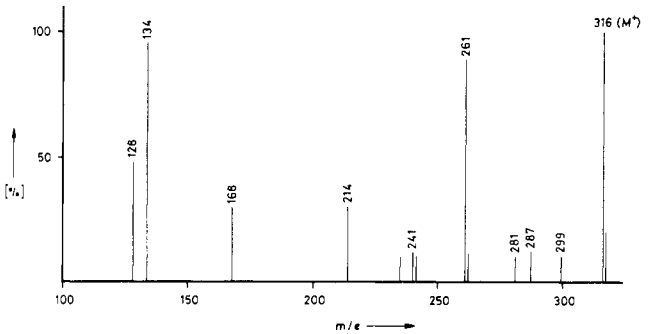

Figure 2. Mass spectrum (significant peaks) of compound **8a** (ion energy, 70 eV; temperature of the sample, 120°, and of the ion source, 160°).

Table IV. Composition of the Volatile Carbonyl Compounds Formed by the Three Lipoxygenases from Soybeans

Compound	Carbonyl compds, mol % L-3 (pH 6.5)	L-2 (pH 6.5)	L-1 (pH 8.5)
Acetaldehyde		1.5	
Propanal	41	46	18
2-*trans*-Pentenal	11	8	5
2-*trans*-Hexenal	9	9	77
2-*trans*,6-*cis*-Nonadienal	2.5		
2-*trans*,4-*cis*-Heptadienal	20	20.5	
3,5-Octadien-2-one	8	8	
2,4,6-Nonatrienal	8.5	7	

L-2 and L-3 form more carbonyl compounds than does L-1 (Table I). This observation correlates with the results of Morita and Fujimaki (1973). These authors have recently shown that in the autoxidation of linoleic acid under mild conditions it is not the monohydroperoxides which are the precursors of the volatile carbonyl compounds, but more polar peroxides of unknown structures.

As shown in an earlier paper (Grosch et al., 1974) only propanal, 2-*trans*-hexenal, and 2-*trans*,6-*cis*-nonadienal are formed in addition to hydroperoxides (main products) when a linolenic emulsion is oxidized by singlet oxygen. The formation of these aldehydes can be explained by the 1,2 addition of the singlet oxygen to the double bonds of the linolenic acid and the cleavage of the dioxetanes formed. A broader pattern of volatile carbonyl compounds which arises during the catalysis of the neutral isoenzymes L-2 and L-3 makes it improbable that singlet oxygen takes part in their formation. However, the range of carbonyl compounds is very similar to that which arises from the autoxidation of a linolenic acid–water emulsion (accelerated by a heme catalyst) (Grosch et al., 1974). The differences in autoxidation come from the reaction velocity, which is higher by at least a power of one hundred in the case of the neutral lipoxygenases, and from the substrate specificity of the enzymes.

It is generally accepted, as a clarification of the autoxidation process, that peroxyl radicals are involved in the formation of volatile carbonyl compounds. We assume that this is also the case for the catalysis of L-2 and L-3. Either some peroxyl radicals generated by the enzymes fragment directly into volatile carbonyl compounds or the very labile polar peroxides which are postulated as precursors of the volatile carbonyl compounds by Morita and Fujimaki (1973) arise from secondary reactions of the radicals. Which reactions of the enzymatically formed radicals in particular occur needs further investigation.

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