Production of 9-hydroperoxy-γ-linolenic Acid by Soybean Lipoxygenase in a Two-Phase System

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9-Hydroperoxy-γ-linolenic acid (9-GOOH) was produced selectively by soybean lipoxygenase (LG) from γ-linolenic acid (GLA) using a two-phase (borate buffer, pH 6.5/hexane) system at a low temperature (10°C) with some anionic surfactants that showed little inhibitory effect on the enzyme at pH 6.5. The system avoided the inhibitory effect of a higher substrate concentration and of hydroperoxide as well as low substrate solubility in an aqueous system. Not lipoxygenase-2 (LG2) but lipoxygenase-1 (LG1) was indicated to be responsible for the production of 9-GOOH. Among the anionic surfactants examined, acetate and sarcosinate were shown to be suitable, but phosphate was not. Ca^{2+} increased the 9-GOOH productivity. The LG1 fraction gave the maximum yield of 35% with 0.5 mM Ca^{2+}, ECT-3N (anionic surfactant, acetate) at 10°C and at 4.8 mM GLA in an emulsion.

Since the first report (1) on the positional specificity of soybean lipoxygenase (linoleate: oxygen oxidoreductase EC 1.13.11.12), many investigations have been done. These results were reviewed by Veldink et al. (2), Gardner (3) and Vliegenthart and Veldink (4).

There are not many investigations on GLA because linoleic acid was used for the substrate in most cases. Among hydroperoxides from GLA, 9-GOOH attracts attention because it serves as a starting material for studies on prostaglandin biosynthesis (5). Hamberg and Samuelsson (6) reported 13-hydroperoxy-γ-linolenic acid (13-GOOH) was the product by soybean LG. But later, Funk et al. (7) prepared 9-GOOH as well as 13-GOOH and showed pH 7 was optimum for preparation of 9-GOOH. Roza and Franke (8) reported "acid LG" (LG1) gave 13- and 9-GOOH in the ratio of about 2:9 at pH 7 and room temperature.

The present report shows the method for 9-GOOH production by soybean LG using a two-phase (aqueous/hexane) system with anionic surfactant and discusses whether LG1 or LG2 is responsible for the reaction.

EXPERIMENTAL

Materials. Soybean LG was purchased from Serva Co. Ltd., and GLA and linoleic acid from Sigma Chemical Co. Ltd. Surfactants except Tween 40 and Triton X-100 were supplied by Nihon Surfactant Ind. Co. Ltd., Tokyo, Japan.

Enzyme assay. Initial velocity of LG activity was measured by the increase in absorbance at 234 nm using a spectrophotometer (Shimadzu UV-300) and a cuvette with a stirrer at 25°C or 10°C. 100-150 μ M sodium γ-linolenate or γ-linolenic acid with surfactant in borate buffer (pH 9.0 or pH 6.5, KH_2PO_4-$Na_2B_4O_7$) with or without 0.5 mM Ca^{2+} was used as the substrate.

Two-phase reaction. In the two-phase reaction, the substrate solution shown in Table 1 was emulsified by homogenization (HA2, Nihon Seiki Ltd.) before the reaction, which gave O/W emulsion. The reaction was started by adding one ml of LG solution (20 mg LG/ml borate buffer at pH 6.5) and mixing the system with a stirrer at 10°C. Reaction time was in the range of 20–160 min. The addition of 0.05 ml of 6N HCl solution to two ml of sample solution broke the O/W emulsion in the case of ECT-3N (sodium polyoxyethylene alkyl ether acetate) and stopped the reaction. GLA and its hydroperoxides were extracted using ethyl acetate. In the case of ECT-3N, the hexane solution separated was used directly for HPLC analysis.

Analysis and separation of GLA hydroperoxides. HPLC (packing material, Finsil-5, Japan Spectroscopic Co. Ltd.; eluent, 2% 2-propanol in hexane with 0.025 % CH_3COOH; UV monitoring at 210 and 234 nm) was used for analysis and separation. Retention time of 9-GOOH was longer than that of 13-GOOH as shown by Chan and Prescott (9) with hydroperoxides of linoleic acid.

Identification of GLA hydroperoxides. Reverse phase TLC (mobile phase: acetonitrile/methanol/water, 6.5:3:0.5, v/v/v) was used to separate the hydroperoxides. The scraped sample was dissolved in methanol and reduced with sodium borohydride for one hr at room temperature. After the reduction, excess $NaBH_4$ was decomposed by acidifying the solution to pH 3.0 with 4 N HCl. The alcohol formed was extracted with diethyl ether and silylated with a mixture of hexamethyldisilazane and trimethylchlorosilane (TMS-HT by Tokyo Kasei Kogyo Co., Ltd.). Mass spectrometry (JMS-D300 by JOEL Ltd.) of silylated alcohol was used in tandem with capillary GLC with diethyleneglycol succinate.

Isolation of LG isozyme. The LG sample was isolated according to the method of Christopher et al. (10) using anion-exchange chromatography. A 100-mg LG sample was applied to a DEAE-Toyopearl 650M column

TABLE 1

Substrate Solution of Two-Phase Reaction

Hexane	10 ml
0.1 M GLA/hexane	1 ml
Surfactant	20 mg
Buffer solution (KH_2PO_4-$Na_2B_4O_7$) (after emulsification)	10 ml
20 mg Lipoxygenase/ml borate buffer (pH 6.5)	1 ml

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(1.6 cm ϕ × 40 cm) which previously had been equilibrated with 0.01 M phosphate buffer (pH 6.8) with 0.5 mM $CaCl_2$. Elution was started (2.5 ml/min) with a linear gradient of NaCl (0–0.2 M) at 4°C. Protein concentration in the elution was monitored continuously by the UV spectrometer at 274 nm. Fractions of five ml were collected and assayed for LG activity at pH 6.5 and pH 9.0.

RESULTS AND DISCUSSION

Figure 1 shows the HPLC chromatogram of separated hexane solution of reaction at pH 9.0 (reaction time, 15 min) and at pH 6.5 (reaction time, 120 min) in a two-phase reaction with ECT-3N. At pH 9.0, GLA already had been consumed at 15 min, whereas at pH 6.5, nearly half of GLA was left even after 120 min.

The main product at pH 6.5 (peak at 19 min of retention time in Fig. 1) was separated by TLC and was reduced and silylated for GC-MS analysis. Figure 2 shows the MS spectrum of the sample. Mass numbers (M/E = 438, 315, 225) indicated the product was 9-GOOH.

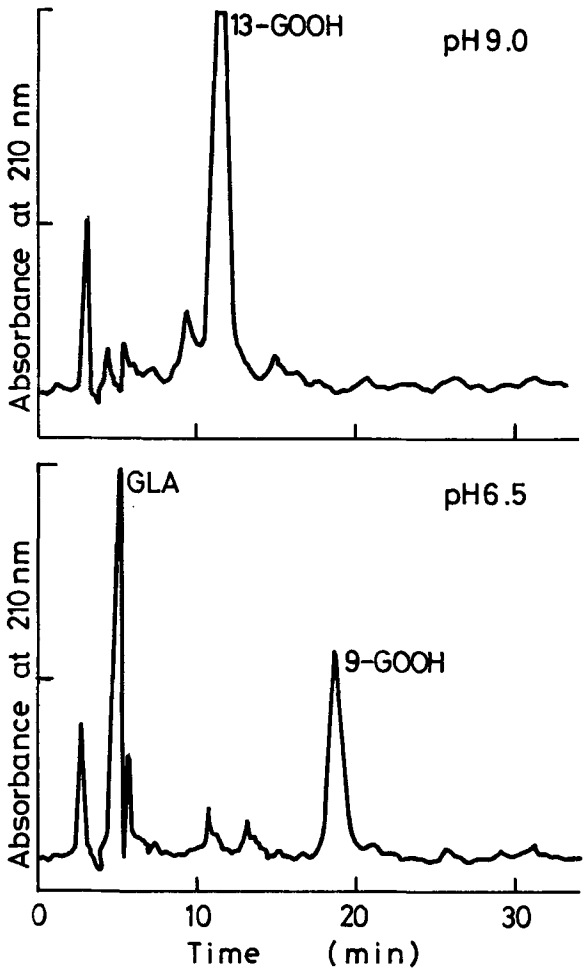

FIG. 1. HPLC chromatogram of separated hexane solution in two-phase reaction with ECT-3N, at pH 9.0 (15 min) and pH 6.5 (120 min).

The main product at pH 9.0 (peak at 11 min of retention time in Fig. 1) increased very rapidly and decreased later (as will be shown in Fig. 3), and it had higher absorbance intensity at 234 nm than at 210 as well as 9-GOOH. Positional specificity of soybean LG at pH 9.0 by Roza and Franke (8) and HPLC behavior indicated the peak at 11 min of retention time to be 13-GOOH. Other GLA hydroperoxide isomers, such as 6-GOOH and 10-GOOH, were not detected.

Table 2 shows the amount of GLA hydroperoxides formed at 10 °C and at various pH's. Reaction times were varied at each pH. Unless the monohydroperoxides decomposed or were oxygenated further during those reaction times at each pH, a kind of average reaction

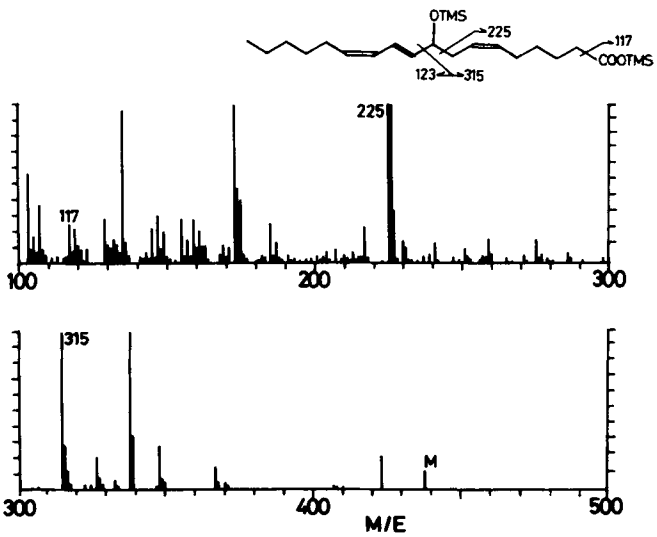

FIG. 2. Mass spectrum of the main product at pH 6.5.

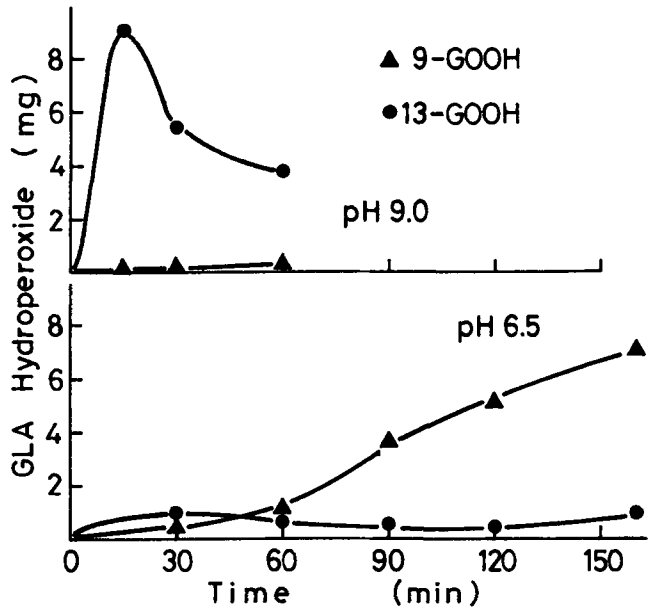

FIG. 3. Time course of the amount of 9-GOOH and 13-GOOH formed.

TABLE 2.

Effect of pH on the Amount of GLA Hydroperoxide Accumulated, Average Rate of Accumulation

pH	Reaction time (min)	9-GOOH accumulated (mg)	Average rate (mg/min)	13-GOOH accumulated (mg)	Average rate (mg/min)	9-GOOH/ 13-GOOH
6.0	160	1.4	0.086	0.74	0.0046	1.9
6.5	160	7.1	0.044	1.2	0.0075	5.9
7.0	30	2.1	0.070	0.26	0.0086	8.1
8.0	15	0.86	0.057	1.1	0.076	0.78
8.5	15	0.58	0.039	4.9	0.33	0.12
9.0	15	0	0	9.3	0.62	0

rate may be defined. This assumption was valid, as will be shown in Figure 3. These average rates and their ratios are also listed in Table 2. The average rate of 13-GOOH formation showed an almost logarithmic increase according to the increase in pH. From the point of average rate of 9-GOOH formation, pH 7.0 seems to be optimum, but at pH 7.0 there was a higher rate of 13-GOOH formation than that at pH 6.5, which was unfavorable for obtaining higher yields of 9-GOOH. As a result, the highest amount of 9-GOOH was gained at pH 6.5.

Figure 3 shows the time course of the amount of GLA hydroperoxides at pH 9.0 and at pH 6.5. 13-GOOH accumulated very rapidly at pH 9.0, but began to decrease at 15 min. This decrease probably came from the further oxygenation of 13-GOOH, which still contained a *cis,cis*-1,4-pentadiene moiety (11). On the other hand, 9-GOOH could not be the substrate of LG and accumulated, consequently, according to reaction time.

As for the temperature effect, 10°C was shown to be optimum for 9-GOOH production. At a higher temperature, the reaction rate of 13-GOOH production increased, and that was unfavorable for 9-GOOH accumulation. Higher dissolved oxygen at a lower temperature may also accelerate oxygen transport and reduce the inhibition of higher substrate concentration (12). The advantage of low temperature for production of hydroperoxide was also reported by Christopher et al. (13) for soybean LG with linoleic acid and Singleton et al. (14) for peanut LG.

Table 3 shows the effect of surfactant in a two-phase reaction. ECT-3N (acetate), ECL-2SYN (acetate), Sarcosinate LN, DDP-8 (phosphate) are anionic surfactants, and TritonX-100 is a nonionic one. Without surfactant, no GLA hydroperoxides were detected after 160 min. High 9-GOOH production was obtained only with anionic surfactants. Among the anionic surfactants examined, acetate and N-acyl amino acid salt (sarcosinate) were shown to be suitable but phosphate was not. In the case of ECT-3N in Table 3, 7.10 mg of 9-GOOH was gained from 27.8 mg substrate, so the yield of 9-GOOH was 25.5%.

In order to clarify the behavior of surfactants, the effect of surfactant addition on the initial velocity of LG was examined in an aqueous solution using sodium γ-linolenate. Table 4 shows the results in terms of

TABLE 3

Effect of Surfactant on the Amount of GLA Hydroperoxides in Two-Phase System

Surfactant added	9-GOOH (mg)	13-GOOH (mg)
Control	0.0	0.0
ECT-3N	7.10	1.20
ECL-2SYN	5.75	0.59
Sarcosinate LN	4.79	0.74
DDP-8	1.06	1.69
TritonX-100	0.19	1.23

Reaction condition, pH 6.5, 10 C, 160 min; control, without surfactant.

TABLE 4

Effect of Various Surfactants on the Initial Velocity of LG

Surfactant	Conc. of surfactant (mg/min)	Relative activity to control pH 6.5 (%)	Relative activity to control pH 9.0 (%)
Control	-	100	100
ECT-3N	0.085	184	76.8
ECT-3N	0.85	21.5	38.1
ECL-2SYL	0.10	63.0	66.5
ECL-2SYL	1.0	91.1	34.1
Sarcosinate LN	0.093	131	81.0
Sarcosinate LN	0.93	113	36.4
DDP-8	0.12	0.7	37.7
TritonX-100	0.10	7.8	73.9
Tween 40	0.10	5.9	78.0

Substrate, sodium-(γ)-linolenate (0.15 mM); control values were 0.27/min (pH 6.5) and 1.18/min (pH 9.0); temperature, 10 C.

Structural formulas of anionic surfactant

ECT-3N: $C_{13}H_{27}O(CH_2CH_2O)_3CH_2COONa$

ECL-2SYL: $C_{12}H_{25}O(CH_2CH_2O)_2COONa$

Sarcosinate LN: $C_{11}H_{23}\overset{\overset{O}{\|}}{C}\text{-}N\overset{\overset{CH_3}{|}}{}CH_2COONa$

DDP-8: $HO\overset{\overset{O}{\|}}{P}\begin{matrix}O(CH_2CH_2O)_4C_{12}O_{25}\\ O(CH_2CH_2O)_4C_{12}O_{25}\end{matrix}$

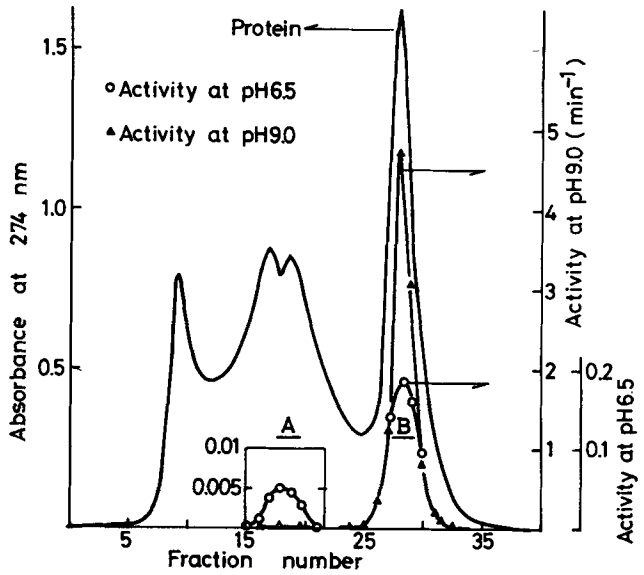

FIG. 4. DEAE toyopearl chromatogram of soybean LG sample with initial activity of each fraction using GLA as substrate at pH 6.5 and 9.0. Fraction A and Fraction B were collected for two-phase reaction.

relative activity to control without surfactant. Two types of the surfactant effect can be seen. The one is for DDP-8, TritonX-100 and Tween 40 (nonionic surfactant), that is, a large inhibitory effect at pH 6.5, but no so large an ihibitory effect at pH 9.0. The other is for ECT-3N, ECL-2SYN and Sarcosinate LN, that is, a small inhibitory effect or even "reverse inhibitory" effect. This apparent activation with low concentration of ECT-3N and Sarcosinate LN may come from improvement of substrate solubility at pH 6.5, as 150 μ M sodium γ-linolenate was not completely solubilized at that pH. A higher concentration of ECT-3N (0.85 mg/ml) decreased the activity greatly. Ability to produce 9-GOOH in a two-phase reaction was directly correlated with the surfactant which kept the initial velocity at pH 6.5.

According to Roza and Franke (8), LG1 was thought to be more suitable for 9-GOOH production than LG2. But from the analogy of pH and Ca^{2+} effect on LG selectivity with linoleic acid (13), LG2 also may contribute to the 9-GOOH production to some extent. In order to make clear whether LG1 or LG2 is responsible for the 9-GOOH production, isolation of a LG sample was carried out by anion-exchange chromatography, the result of which is shown in Figure 4. The reaction of Fraction A was accerelated by Ca^{2+} and bigger at pH 6.5 than at pH 9.0, and the product of linoleic acid was 9-hydroperoxy linoleic acid as well as 13-isomer. This indicated Fraction A to be LG2. And Fraction B was thought to be LG1 from the similarity to the result of Christopher et al. (10). Each fraction was examined in a two-phase reaction system, with and without Ca^{2+}. 9-GOOH was not produced by LG2, but was produced by LG1, contrary to the analogical prediction of LG1 and LG2 to linoleic acid. These results show agreement with the data shown by Roza and Franke (8), that is, LG1 is responsible for the formation of 9-GOOH at neutral pH. Addition of Ca^{2+} increased the productivity of 9-GOOH.

The advantage of a two-phase reaction system lies in two points. Those are avoidance of inhibition by substrate (15-17) and by product hydroperoxide an (18) as well as high solubility of the substrate in hexane. In an aqueous system, substrate solution must be the order of one mM at most, to get rid of high substrate inhibition and also because of its low solubility. Maximum yield of 9-GOOH with 0.5 mM Ca^{2+} by Fraction B was 35%, which may not seem to be high enough, but high GLA concentration in hexane can compensate for low yield in terms of the amount of 9-GOOH obtained. The mechanisms of anionic surfactant action in this system are to be examined.

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